Niche differentiation within the *Wiseana* (porina) species complex: pasture pests of New Zealand

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Sylvester Richard Atijegbe

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The most important sector of New Zealand's primary industries is pastoral agriculture, but the production and quality of the pasture itself is threatened by porina moths. Porina is a complex of species belonging to the genus *Wiseana* (Lepidoptera: Hepialidae) which is endemic to New Zealand. The larvae are major pests of exotic pastures, particularly in the central and southern North Island and most of the South Island of New Zealand. Despite this impact, no such damage of any New Zealand native plants has been ascribed to these insects and, to date, no studies of porina feeding on native plants have been done to understand this discrepancy. However, to develop more stategic approaches to improve management of porina in pastures, it is critical to understand why porina have moved into exotic pastures and become so successful, relative to what the populations must have been in their native habitats.

Here, fundemental information is developed to unravel key aspects that may be linked to porina's success. In particular, to confirm that porina do indeed develop and survive better on exotic compared to native host plants, determine what the identities are of the species that have moved into pastures and if there are differences in their seasonal flight patterns. Behavioural studies are needed to observe larval feeding and survival on exotic and native host plants under controlled conditions, and to determine if any differences may be associated with the relative nutrient content of those hosts. Such information may then enable hypotheses to be developed as to which New Zealand native plants are, or were, likely hosts for these species, as well as to what might have been the evolutionary mechanism to move from those hosts to exotic pasture plants, i.e. host range expansion or host switch. Finally, if egg-larval development data can be developed, can this be used to build preliminary forecasting models that might enable the best timing of application of control measures in the field be determined.

Results of the experimental work to address these knowledge gaps showed with a pilot study at the outset that larval development and survival of field-collected porina species on native and exotic plants showed mortality from 13% to 75% and larval survival time from 96 to 177 days (Chapter 2). Subsequently light trap catches showed *W*. *cervinata* and *W*. *copularis* to be the two main species infesting pastures in Canterbury, with *W*. *cervinata* flying from late spring to early summer and *W*. *copularis* flying from early summer to late summer (Chapter 3). A simple method was developed that improved laboratory rearing and survival of porina larvae from eggs by 90%, and revealed that eggs of *W*. *cervinata* at 15 and 22℃ hatched earlier compared to *W*. *copularis* and *W. umbraculata* (Chapter 4). Larvae of *W*. *cervinata*, *W*. *copularis* and *W*. *umbraculata* reared from eggs further confirmed the differential success of their growth and development, being significantly better on exotic (*Trifolium repens* and *Lolium perenne* × *Lolium multiflorum*) than native (*Aciphylla squarrosa, Festuca actae*, *Chionochloa rubra*, *Poa cita* and *Phormium tenax*) plants and with implications for species fitness, population dynamics and management (Chapter 5). The relative nutritional status of these plants was measured and over 100 metabolites were detected in each plant species (Chapter 6). Of these metabolites, which were classified into 11 groups (amino acids, cyclitols, fatty acids, organic acids, other N-compounds, sugars, sugar acids, sugar alcohols, phytosterols, miscellaneous and unknowns), N and silica were shown to be higher in the exotic host species, but C, CN ratios and fibre content were higher in the native host plants. Observations on the foraging behaviour of *Wiseana* larvae over a three day period showed that *W. copularis* were more active grazers, creating multiple burrows as they forage with 63% of larvae emerging to feed, compared to *W. cervinata* creating a single burrow and 50% of larvae emerging to feed (Chapter 7). However the locomotory ability of the pupal stage to move up and down the burrow showed that *W. cervinata* descended slightly faster than *W. copularis* in response to stimuli, suggesting it may have a better ability to avoid predators (Chapter 8). Together, the prediction of peak flight times, and the time when early instar larvae are vulnerable at the soil surface can help researchers and farmers to assess porina population dynamics more effectively for the application of management decisions. Lastly, data on porina development, seasonal flight and weather were used to design a simple prototype model for predicting the best timing for the application of pesticides.

Overall, the approaches and results obtained in this thesis have provided essential biological evidence to help understand that a mixture of host range expansion and host switching mechanisms may have contributed to the success of *Wiseana* spp. in New Zealand pasture. It has also helped to identify components of their phenology that can be used in improved management stategies. The conclusions offer new research directions for the investigation of the *Wiseana* invasion processes in general.

Key words: Porina, *Wiseana copularis*, *W. cervinata*, *W*. *umbraculata*, rearing method, pupal movement, burrow, plant metabolites, foraging behaviour, metabolites, nitrogen, exotic and native

host plant, host expansion, linear and nonlinear models, degree-day, phenology, lower threshold, weather data, peak flight, prediction.

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Daddy! I finally got it done, just as I assured you I would.

Rest in perfect peace.

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Chapter 1

General Introduction

1.1 Background

Pastoral agriculture is the most important of New Zealand's primary industries worth an estimated $19.5 billion per annum (Ferguson *et al*. 2019). Pasture production based on introduced grasses and clovers has been both profitable and resilient, but is particularly susceptible to pest outbreaks from several major insect pests (Zydenbos *et al*. 2011, Jackson *et al*. 2012, Ferguson *et al*. 2019). One of these is porina, which is actually a complex of endemic species belonging to the genus *Wiseana* (Lepidoptera: Hepialidae, Nielsen & Scoble 1986). The larvae are major pests of pastures in the central and southern North Island and most of the South Island of New Zealand (Dumbleton & Dick 1941, Cottier 1962, Helson 1966, Helson 1974, Kalmakoff & Longworth 1980, Scott 1984, Pottinger *et al*. 1987, Barratt *et al*. 1990, Dugdale 1994, Jensen & Popay 2004, Popay *et al*. 2012, Ferguson *et al*. 2019), the damage from which is estimated to cost $172 million p.a. (Ferguson *et al*. 2019). However, before the advent of European agriculture in New Zealand, forest covered large areas (Miller 1929) and porina probably inhabited relatively small areas of native grasses and herbs which may have limited their abundance (Lowe 1973). Subsequently, European agricultural practices appear to have favoured at least some porina species and allowed them to colonise pastures. They are now able to exploit several exotic plant species such as white clover and ryegrass, which appear very favourable to their development (Kelly 1971, Kain 1980, Barratt *et al*. 1990, Fenemore 1982, Jensen & Popay 2004) and susceptible to porina feeding (Jackson *et al*. 2012). In contrast, in porina's natural environment they are controlled by several naturally occurring pathogens (bacteria, fungi and viruses) (Crawford & Kalmakoff 1977, Barlow *et al*. 1986) such that the populations are kept in comparatively low numbers (Lowe 1973) because their natural habitats are not cultivated. *Wiseana* have moved into cultivated plantings because the natural population regulation mechanism is disrupted by pasture cultivation and this has been an important factor in them becoming pests and a major driver of damage in young pastures.

Scientists and farmers have a long history with porina management, but this has mostly focused on the pest's biology and short term impacts (Zydenbos *et al*. 2011). Presently, the use of insecticides is becoming increasingly unpopular among farmers, consumers and

regulators due to their environmental impacts, and with the ban of diazinon (approved insecticide for porina control) coming into effect in 2028, there has been a shift away from broad spectrum insecticide management towards more environmentally benign control methods such as stock management (Stewart & Ferguson 1992), insect growth regulators (Ferguson 2000), optimisation of endophyte infected pastoral grasses (Jensen & Popay 2004, Easton & Fletcher 2007, Ferguson *et al*. 2019) and development of microbial pesticides (Hajek *et al*. 2009). To maximise the effectiveness of these alternative strategies it has become apparent that a greater understanding of *Wiseana* phenology, development and flight times, and community composition is required. Although mitigation strategies exist, their large scale adoption by farmers still faces challenges. For example, the insect growth regulator (IGR) diflubenzuron, is most effective when applied against the small, frequently moulting larvae (Ferguson & Crook 2004). The presence of early instar larvae is determined from the time when moths fly and lay eggs and this is in turn is affected by species and population haplotype. Effective use of diflubenzuron against a particular life stage requires monitoring and knowledge of *Wiseana* biology.

1.1.1 Plant landscape of New Zealand prior to human settlement

Most of the pre-Polynesian (Maori) landscape of New Zealand was clothed in temperate rain-forest, with a few exceptions (Ogden 1998). Despite natural ignition sources such as lightning and volcanism (Molloy *et al*. 1963), the forests rarely burned prior to the arrival of people (Burrows 1990, Wardle 1991). The vegetation of the North Island was dominated by large areas of podocarp/mixed hardwood forests and scrublands as well as fernlands and swamplands. There were also small areas of beech and beech/podocarp forest, coastal strips of dunelands and tussock grassland on the central plateau (Brougham 1978). The South Island on the other hand was quite different, with large areas of lowland tussock grasslands and only very small areas of swamplands, fernlands and scrublands. The lowland tussock grasslands gave way with elevation to sub-alpine grasslands and to a lesser extent, scrublands, and in the higher reaches of the Southern Alps, to the alpine barrens. On the western side of the Southern Alps there were large areas of beech/podocarp forests and a lesser area of podocarp/mixed hardwood forests. There was also some beech on the eastern side of the Southern Alps (Brougham 1978).

The arrival of Maori colonists approximately 700 years ago used fire to rapidly eliminate forest over large areas of the lowlands (McGlone 1983). This may have given rise to the tussock

grasslands in the South Island which, being amongst their natural hosts, may have benefited porina populations (Basher *et al*. 1990, Douglas & Allan 1992). Also, pastures did not exist in New Zealand pre-European colonisation. The European agricultural practices of using exotic pasture plants in livestock production systems seem to have assisted porina to colonise pastures.

1.1.2 Present day plant landscape

In the last 100 or so years, the vegetation of New Zealand has been dramatically altered, and in a land area of approximately 26 million ha, approximately 13 million ha are now farmed at varying levels of pasture development. The remainder is mostly mountainous and still comprises more than 6 million ha of forested lands (endemic and exotic), about 6.6 million ha of the other non-forested lands (scrub, second growth, wastelands, barren lands, cities, roadways, etc.), and about 0.4 million ha in lakes and rivers, etc. Thus more than 95% of the country's farmed lands are devoted to pastoral farming, nearly two-thirds of which is in sown pastures, and the remainder mainly tussock grasslands (Encyclopaedia of New Zealand 1966). The main use of pasture in New Zealand is for dairy farming which in 2014/2015 used 1.75 Mha of high-producing exotic grassland (Anon 2016).

1.1.3 Classification and identification of *Wiseana*

Hepialidae in New Zealand comprises 27 endemic species in the genera *Aenetus, Aoraia, Cladoxycanus, Dioxycanus, Dumbletonius, Heloxycanus* and *Wiseana* (Brown 1998). They form part of the Hepialidae *sensu stricto* (Nielsen & Scoble 1986), one of 12 monophyletic families within the superfamily Hepialoidea (Nielsen 1989). An overall picture of hepialid relationships in the Southern Hemisphere has not yet emerged as only the southern South American fauna (Nielsen & Robinson, 1983), the primitive *Fraus* from Australia (Nielsen and Kristensen 1989) and the New Zealand fauna (Dugdale, 1994) have been comprehensively examined. Dugdale (1994) carried out a taxonomic revision of the New Zealand family based on morphology and the taxa fell into four distinct groupings with characteristics affiliated with taxa in Australia and further afield. Consequently, he proposed four informal lineages for the New Zealand fauna, the *Aenetus* lineage, *Aoraia, 'Oxycanus' s. str.* and *'Oxycanus' Cladoxycanus*.

The genus *Wiseana*, is well known to systematists and applied scientists in New Zealand because it had proved difficult prior to 1994 to establish the number and distribution of species, and to find characters to identify the species causing damage (Brown *et al*. 2000). The

genus has had a turbulent systematic history because the adults have overlapping intra-specific and interspecific morphological characters, distributions and flight times, and the larvae have no distinguishing morphological characters (Brown *et al*. 1999). Studies by Herbert (1995) and Brown *et al*. (1999, 2000) suggest possible explanations for the historic difficulties of species identification within the *Wiseana* complex. Due to these difficulties, the actual species involved in research prior to this period are in doubt, either because the species is referred to as *Wiseana* spp. or, if the species is named, the identification is now in doubt (Stewart 2001, Ferguson 2004). However, with the advent of the scanning electron microscope (Archibald 1984), molecular technologies, (MacArthur 1986, Herbert 1995, Brown *et al*. 1999, 2000) and detailed morphometric studies by Dugdale (1994), the *Wiseana* complex has been confirmed to have seven distinct species being *W*. *cervinata* (Walker), *W*. *copularis* (Meyrick), *W*. *fuliginea* (Butler), *W*. *jocosa* (Meyrick), *W*. *mimica* (Philpott), *W. signata* (Walker) and *W*. *umbraculata* (Guenée), all endemic in New Zealand. Brown *et al*. (1999, 2000) further identified population haplotypes for *W*. *cervinata, W*. *copularis* and *W*. *signata*. Recently, a simpler and cost effective polymerase chain reaction restriction fragment polymorphism (PCR-RFLP) diagnostic test for *Wiseana* species developed by Richards *et al*. (2017) has further reinforced this species-level classification. With improved confidence in the species distinctions, the most recent research suggests that not all of the species cause damage to pasture and that while some may cause only geographically localised damage (*W*. *cervinata*, *W. mimica* and *W*. *jocosa*), others cause regional (*W*. *copularis*) damage (Barratt *et al*. 1999). Herbert (1994) and Richards *et al*. (2017) have also shown that several species can co-exist in pastures.

1.1.4 A general biology and life cycle of *Wiseana*

Wiseana moths (Figure 1.1) are holometabolous insects. They are univoltine and fly during spring, summer and autumn with flight peak dates and periods which vary with weather (French 1973), species/haplotype (Ferguson *et al*. 1999, 2016), locality and year (French & Pearson 1979, Carpenter *et al*. 1980). The moths are capable of long flights and are very fecund (French 1973, Jackson *et al*. 2012), with a single female producing over 3,000 eggs (Stewart 2001, Jackson *et al*. 2012, Ferguson *et al*. 2019). Porina eggs are ovoid, cream in colour when laid, but turn grey and finally black within ten hours (Quail 1900). Depending on the species and temperature, eggs hatch in about 3-6 weeks (Dumbleton 1945, French & Pearson 1979, Fleming *et al*. 1986, Ferguson & Crook 2004, Atijegbe *et al*. 2017). The newly

hatched larva is creamy-white with a large, pale brown head. Seven weeks after, the larva is a thinly formed larvae approximately one centimetre in length (Quail 1900). At this stage the three pairs of thoracic legs, and five pairs of abdominal legs are clearly visible (Doull 1951) and the small larvae shelter in plant debris, under silken shelters or in camouflaged soil depressions (Pottinger 1968) close to the soil surface for 6-8 weeks (French & Pearson 1981, Barlow 1985, Fleming *et al*. 1986, Stewart 2001). Thereafter, the larvae construct noticeable vertical tunnels 15 - 30 cm deep (Kelsey 1951, Pottinger 1968). As the larvae grow they form permanent burrows in the soil which increase in depth with larval age and size until they reach the sub-soil or, in very deep light soils, a depth of approximately 100 cm. Larval development is temperature related (Ferguson & Crook 2004) and they remain as larvae in the soil for about nine months (Barlow *et al*. 1986), growing in size before pupating. A mature larva (Figure 1.2) is soft and flabby up to 8 cm in length, dark grey-green dorsally and cream underneath and the obtect pupae is 3 cm long, a dark straw colour with reddish spines (Quail 1900).

The male pupa is generally smaller than that of the female with a much closer spacing of the anal and genital openings than the female (Waller 1966). The adult is a heavily built moth up to 2 cm in length with a wingspan of 2.5 - 5 cm (Relson 1966). The wings have markings that are not consistent within species nor distinctive between species with the exception of *W*. *signata* and *W*. *umbraculata*, but their predominant colours are brown, black, and brownish yellow (Atkinson 1956). The antennae of the two sexes are quite distinct, plumose in males and filamentous in females. The male is smaller than the female, with the body slightly tapering to the end of the abdomen. The female body is engorged with eggs and more cylindrical than that of the male (Kelly 1971). Mating occurs almost immediately after the females emerge and before they are capable of flight, taking 2-15 minutes and oviposition commences almost immediately (Dick 1945). They then crawl and fly amongst the foliage laying eggs in groups of up to eight at a time. They may then fly 200 - 300 cm, come to rest and lay more eggs, at a rate of up to five eggs every four seconds (Dick 1945). The number of eggs laid per female varies widely and Pottinger (1968) noted that fecundity is correlated with larval nutrition: moths from a starved population producing less than half as many eggs as those from a well fed population. The adults have severely reduced mouthparts and do not feed. They live from one to four days, on average (Dick, 1945). Dumbleton (1945) stated that fifty percent are dead after three days, and one hundred percent after four. The females

usually die soon after laying their eggs. But Pottinger (1968) has observed females living to nine days and males to seven days.

Figure 1.1: Adult *Wiseana* moth (Photo Source: AgPest, New Zealand**)**

Figure 1.2: Mature *Wiseana* larva (Photo Source: T.E.R:R.A.I.N - Taranaki Educational Resource: Research, Analysis and Information Network, New Zealand)

1.1.5 Larval food intake and feeding behaviour

The young larvae are thought to feed on microflora (Grehan 1989) but they will also feed on live plant material immediately on hatching (Ferguson *et al*. 2003). They show rather unusual behaviour because the larvae live in burrows underground and emerge only at night to feed, either in situ at plant bases (Colin Ferguson, personal communication, AgResearch, New Zealand) or harvesting plant leaves to take back to their burrows (Pottinger 1968, Farrell *et al*. 1974, French & Pearson 1981). They prefer to remain in contact with burrow entrances while feeding, consequently removing all herbage nearest the burrow entrance before having to venture completely out of their burrows. In this way the feeding areas increase as the larvae increase in size and the plants immediately adjacent to burrows are often over grazed and killed (Barlow 1985). As feeding progresses, surface runways are often constructed to the feeding sites from the tunnel entrances (Kelsey 1951, Grehan 1989) which are camouflaged by silken threads, faecal pellets, excavated soil particles and surface debris (Pottinger 1968).

Dugdale (1994) listed *W. cervinata, W. fuliginea*, *W*. *mimica* and possibly *W. copularis* as damaging species, but did so speculating on the basis that the large final instar larvae of these species competed with stock for spring pasture production. Generally, the species considered responsible for most pasture damage are *W. cervinata* and *W*. *copularis* based on abundance of porina moths measured during flights (Ferguson & Crook 2004, Ferguson *et al*. 2019). Recent research where larvae in pastures have been identified (Richards *et al*. 2017, Ehau Taumanu 2017, Mansfield *et al*. 2017) supports that contention while confirming other species are occasionally found. Porina has been observed as a pest in native tussock grasslands (*Chionochloa rubra*, Kelsey (1968) and *Festuca novae-zelandiae*, C. Ferguson personal communication, AgResearch, NZ) with only two other records of porina species causing damage in other non-pasture habitats: Miller (1952) recorded possible porina damage to kumara (*Ipomoea batatas*) (although this was debunked by Ehau-Taumanu 2017) and Miller (1971) recorded *W. signata* feeding on flax (*Phormium* sp.). French & Pearson (1981) found that in a population of *Wiseana* larvae, an average of only 50 per cent were feeding on any one night and feeding was suspended by individual larvae for periods that sometimes exceeded 10 consecutive nights. This agrees with the findings of Esso (1970) that rainy, frosty and dry conditions inhibited feeding and stated that ecdysis was the limiting factor to feeding. Several studies on feeding by *Wiseana* show that larvae exhibit different host preferences in relation to species diversity and combination of host-plants (Harris 1969, Harris & Brock 1972, Farrell *et al*. 1974). Larvae begin feeding in early autumn and continue throughout the late autumn and winter causing damage to pasture (French 1981). Using head capsule width as an index, Dumbleton (1945) noted that the most rapid growth period corresponds with severe host plant defoliation.

Harris (1969) studied the effect of larval feeding on mixed pasture species in an unheated glasshouse. He found that larval survival was highest under white clover and least under ryegrass, with an average food intake of 0.0321 g dry matter (DM) per larva per day over 133 days. The average liveweight of the surviving larvae was 1.102 g representing a liveweight gain of 0.808 g. Allen (1968) calculated food intake as 0.015 g DM per larva per day over an 89 day period in spring for larvae having an average liveweight of 0.486 g. French (1981) too showed the average nightly intake of white clover (green weight) for a healthy larva increased from 0.0078-0.0340 g in four months. Farrell *et al*. (1974) however, found that the amount of food eaten was less than that estimated by Allen (1968) and Harris (1969) over similar periods and

he attributed this to differences in the experimental environment, in techniques of measuring feed intake, and in the method of calculation

1.1.6 Adult flight

On the evening of emergence, the pupae protrude half way out of the tunnel entrance, the mid-dorsal line splits, eclosion occurs (Dick 1945) and the teneral adult slowly expands its wings and crawls up a grass blade before flying (Pottinger 1968). In general, the change from cold to milder spring conditions increases the numbers flying (Cumber 1950). Dumbleton (1945) maintained that the key factor governing the numbers in flight was temperature. However, Dick (1945) could find no correlation between flight and temperature and French (1975) indicated that atmospheric pressure/impending rain and calm nights preceded flights.

Various authors such as Dumbleton (1945), Cumber (1951), Cottier (1962), Gaskin (1964) and Helson (1966) have shown that *Wiseana* species flight varies between regions and from year to year. The severity of porina attack in a given location is often related to fluctuations in climatic conditions over a period of time (Kelly 1971). However, the identities of the species mentioned in these early studies are in doubt following the revision of the *Wiseana* genus (Dugdale 1994).

In Otago and Southland, where species complexity is greatest and porina achieves its greatest significance as a pest, the predominant species, *W. copularis* and *W*. *cervinata*, begin flying in October, with a peak flight in November-December for *W*. *cervinata* and that for *W*. *copularis* in January (Ferguson *et al*. 1999). In the West Coast, *Wiseana* spp. flight activity begins in October through to March with a peak from early December to mid-January (Mansfield *et al*. 2017).

1.1.7 Optimising pest management of *Wiseana*

For the integrated pest management (IPM) approach used to control porina to be successful, precise timing of the spray applications of the IGR diflubenzuron is crucial as it is only effective with the earlier larval instar stages. More precise timing reduces the number of applications, cost and potential for pesticide resistance to develop as well as supporting sustainable or IPM practices. However, as close monitoring and observing population development in the field is impractical and time consuming, a robust timing model would assist farmers to both monitor and schedule control measures. For example, knowing when porina start flying and when peak

flight occur would help farmers be more prepared and to assess more precisely insect population development for the application of chemical or cultural control measures. Forecasting models have been developed for many insects (Collier & Finch 2001) to predict emergence times (Bentz *et al*. 1991), distribution (Fand *et a*l. 2014), outbreaks (Delatte *et al*. 2009), and voltinism (Kroschel *et al*. 2013), with applications including pest management, epidemiology, forensic science, mass insect rearing, and conservation plans (Roy *et al*. 2002, Kontodimas *et al*. 2004, Moore & Remais 2014, Chuine & Régnière 2017). Many of these models are based on accumulated degree days (e.g. Eckenrode & Chapman 1972, Butts & McEwen 1981, Wold & Hutchison 2003, Schaub *et al.* 2005, Skinner *et al.* 2006, Damos & Savopoulou-Soultani 2010, Tran *et al*. 2012). Degree day models in particular can play an important role in IPM by providing a simple means of predicting insect development, and therefore providing more precise timing for implementation of control activities. Modeling insect pest dynamics and management options facilitates the search for the best management strategy as models help summarize relevant known aspects of a pest's biology and ecology, highlight uncertainties, and assess and prioritize potential control strategies through 'computer experiments' for field testing (Shea *et al*. 2006). Models also allow us to ask questions that are hard or even impossible to answer in the field (Shea *et al*. 2006). Modeling approaches, if used correctly, can save valuable time and resources.

1.2 Aims and objectives

A major criticism of much of the literature on porina in New Zealand is that in over a century of pasture research only a few articles mention the native host range of porina and none of these articles actually worked on any of the native hosts mentioned. The reason might be that during this period, research was geared towards the elimination of porina rather than understanding why or how they have become pest on exotic pastures in the first place. Based on the stark changes in plants inhabiting much of the New Zealand landscape since human settlement, one pausible hypothesis is that, the success of porina in occupying the niches created by these exotic pastures may be as a result of those plants being more suitable than the original native habitat and presumably the absence of defences in the exotics. This may be in terms of their nutritional value, naïve plant defences, intensive cultivation of the plants making them more available and the absence of evolved natural enemies of porina in exotic pasture.

This study took an ecological approach in an attempt to answer the following questions. Why has porina become a pest on exotic pastures? Was it due to the absence of the natural enemies found in its native habitat, caused by agricultural activities of the early Europeans which still persist today, or was it the nutritive quality of the introduced exotic plants themselves? Also, do different species of porina show different feeding patterns and flight times as behaviours that could affect control efforts? Why have *W*. *cervinata* and *W*. *copularis* apparently adapted to exotic pastures better than the other species and do they actually occupy the same niche and finally, "Why has porina become invasive in New Zealand pastures?

The aims of this thesis were to answer the above questions. These aims were achieved by comparing *Wiseana* spp. interactions with putative native hosts and exotic pasture plants to:

(i) Investigate the development of immature life stages of *Wiseana* spp. to adult emergence (Chapter 2).

(ii) Investigate the feeding preferences and survivorship of porina species (Chapters 4 and 5).

(iii) Investigate the nutritional differences between putative native hosts and exotic pasture plants (Chapter 6).

(iv) Investigate the timing, duration and periodicity of feeding and amount eaten by porina species under controlled conditions (Chapters 4, 7 and 8).

Also with just the exotic pasture plant species to:

(v) Develop a degree day model (Chapter 10) from novel phenology data (Chapters 3 and 9) to achieve more precise timing for implementation of control activities for *W. cervinata* and *W. copularis* IPM.

1.2.1 Structure of the thesis

This thesis represents work commenced in February 2015 under the supervision of Dr Sarah Mansfield, Dr Michael Rostás, and Assoc. Prof Sue Worner, with Mr Colin Ferguson as an adviser and assistance from Dr Karen Armstrong. The thesis consists of nine experimental chapters, each assessing the evidence of differences between the species that contribute to

the overall aims of the thesis. The work has been written as scientific papers to journals, some already published and others in preparation for submission and is reflected in the stylistic differences between chapters. In this context, the structure of the thesis addressing the Objectives above is as follows:

Chapter 2 - *Growth, development and survival of porina* (*Wiseana species*) *on selected native and exotic grass species in New Zealand*. A pilot experiment gathering preliminary data on the development and survival of unidentified, field collected porina larvae on some native and exotic plants.

Chapter 3 - *Seasonal flight and identification of Wiseana species in Canterbury and implications for their management.* This study describes how data from monitoring a simple light trap can be used to determine when to apply the most effective control strategy, and is slightly different for different *Wiseana* species.

Chapter 4 - *Laboratory handling and rearing of early instar porina*. A simple and cost effective technique for rearing *Wiseana* in the laboratory is described for the purpose of enabling subsequent behavioural studies under controlled conditions.

Chapter 5 - *Development, preference and host shift of porina on exotic and native plant hosts*. Here, the mechanism underpinning the success of *W. copularis* and *W*. *cervinata* on pastures is considered and suggests a combination of host range expansion and host shift has occurred for different species.

Chapter 6 - *Nutrient content and metabolite profiles of host plants of porina.* This chapter investigates the nutrient content and metabolite profiles of native and exotic pasture plants tested against *Wiseana* spp. (Chapter 5) and concluded that differences in the chemical composition of the of the plants, particularly nitrogen, may have played a role in the success of *Wiseana* in pasture.

Chapter 7 - *Foraging behaviour of Wiseana larvae.* The nocturnal larval foraging behaviours of *W. cervinata* and *W*. *copularis* were observed and the sequences quantified in the laboratory under infra-red conditions, enabling a discussion on the implications of foraging differences in pest management.

Chapter 8 - *Locomotory ability of Wiseana pupae.* The unique movement of pupae of *W. cervinata* and *W*. *copularis* is described in artificial burrows, and reveals how this movement enables them to evade extreme/unfavourable weather conditions and potential predators.

Chapter 9 - *Thermal requirements and degree-days for Wiseana species.* Using the developmental data for *W. copularis* and *W. cervinata* (Chapters 4, 5 and historical data) two linear models were compared and, based on goodness of fit, the Ikemoto-Takai model was selected because it had the better fit and lowest standard error to provide the critical parameters of the lower temperature threshold for development (T_0) and degree-days (DD) for egg-larval development required for the design of phenology models (Chapter 10).

Chapter 10 - *A phenology model for predicting the optimal time for applying measures for the pasture pest Wiseana in some parts of New Zealand.* A prototype forecasting model to estimate the optimal control period for *W. copularis* and *W. cervinata* is based here on physiological time in the form of degree days (DD). This include how T_0 and the thermal constant or sum of effective temperatures (SET) were used in combination with historical light trap and weather data to develop a timing model for peak flight to predict the optimal time to apply control is discussed.

Chapter 11 - *General discussion and conclusions.* Evidence for niche differentiation between the *Wiseana* species from the various studies in this thesis and the literature are synthesised into a whole, culminating in further questions and recommendations for application of the knowledge in terms of improved pest management as well as for future research**.**

These chapters represent papers co-authored with my supervisors Dr Sarah Mansfield, Dr Micheal Rostás, Assoc. Prof. Sue Worner and Mr Colin Ferguson as adviser. Mr Ferguson and I collected all the specimens, I carried out all of the laboratory work, data collection, statistical analyses and writing of the papers. The co-authors provided advice on methodology and analysis, and, together with Dr Karen Armstrong, provided guidance on writing style and editing.

Chapter 2

Growth, development and survival of porina (*Wiseana* species) on selected native and exotic grass species in New Zealand.

Results of this chapter published as

S. R. Atijegbe, S. Mansfield, M. Rostás, S. Worner and C. Ferguson (2017). Growth, development and survival of porina (*Wiseana* species) on selected native and exotic grass species in New Zealand. In: *Invertebrate Ecology of Australasian Grasslands. Proceedings of the Ninth ACGIE* (ed. S.N. Johnson), pp. 184-187. Western Sydney University, Hawkesbury, NSW, Australia.
https://docs.wixstatic.com/ugd/962e97_7f9bcc7c3aaa4b6583b5e3f334bbc6d8.pdf

2.1 Abstract

Porina (*Wiseana* spp.), are endemic insects that are major insect pests of pasture in New Zealand. Despite their impact on exotic pastures, porina are not known to cause significant damage in their native habitats. To improve management of porina in pastures it is important to determine why porina cause considerable damage in exotic pastures but not in their native habitats, and requires an understanding of their growth, survival and preference for native or exotic plant species. To date, the feeding behaviour of porina has not been studied in detail on native grasses, and there are also questions about how well porina larvae develop on native plant species. The growth rate and feeding behaviour of porina larvae was studied when supplied with foliage from several native and exotic plant species: *Festuca actae, Acyphylla squarrosa*, *Poa cita*, *Chionochloa rubra*, *Phormium tenax*, *Lolium perenne* × *Lolium multiflorum*, and mixed grass species which consists of fescue, ryegrass and weeds. Mortality, larval survival time, growth rate, and the relative growth rate of larvae ranged from 12.5–75%, 96–177 days, 57–225% and 0.0057–0.0083 (gm/gm/day) respectively. This study confirmed the success of porina larval growth and development was less on native compared to exotic plant hosts that will have implications for individual fitness, population dynamics and potential management.

Key words: Apiaceae, exotic, host plant suitability, native, Poaceae, Asphodelaceae

2.2 Introduction

Pasture is a major component of livestock and dairy production in New Zealand. New Zealand pastoral agriculture is based on a composition of introduced grasses and clovers that has been both profitable and resilient but is particularly susceptible to pest outbreaks (Jackson *et al*. 2012). In 2015, white clover and ryegrass contributed about NZ$19 billion to the New Zealand economy. These high quality pastures are being threatened by two major insect pests. One of these pests is the *Wiseana* complex (Lepidoptera: Hepialidae) of seven recognised endemic species, commonly known as porina. Several of these species are major pests in pastures particularly in the southern North Island and many parts of the South Island of New Zealand (Popay *et al*. 2012). Porina moths are univoltine and fly during spring and summer with a mean flight peak date and periods which vary with locality and year (Carpenter *et al*. 1980). Porina larvae feed mainly on pasture plants – exotic ryegrass and clover and show rather unusual behaviour because the larvae live underground in burrows, emerging only at night to feed on the pasture. The larvae remove all herbage nearest the tunnel entrance by severing tillers, leaves and stems near ground level and dragging foliage into their tunnels where they are eaten (French & Pearson 1981). During this period they remain in the soil for about 4–9 months feeding before pupation. It is these subterranean larvae which cause pasture damage during autumn, winter and spring, depending on the locality and year. It is hypothesized that porina development is more successful on exotic pasture species than on native plant species.

Some authors have recorded porina feeding on a broad range of native plants (see various authors in Spiller *et al*. 1982, White 2002). However, none of the supposedly native plants have been specifically studied as hosts (ability of insect larvae to complete development into viable adults) of porina, and there are questions about the larvae feeding on these plants. Knowledge of porina larval development is essential because it can help to explain how some porina species have adapted to exotic pastures. This study examines the development success of porina larvae on native and exotic plant hosts. Such knowledge could provide useful information for the management of porina in pastures.

2.3 Methods

2.3.1 Insects and plant material

Porina larvae of mixed ages were collected from porina infested paddocks. The larvae were placed into individual 120 ml plastic containers which were half to three quarters filled with 3-4 months old potting mix and with a hole drilled in the cap for air flow. To check for disease symptoms prior to the development experiment, the larvae were kept in a constant temperature (CT) cabinet at 15°C, reverse daylight (12 L: 12 D), and fed mixed grass species (containing fescue, ryegrass and lawn grasses) every 2-3 days for one month. Only healthy larvae that survived this initial screening were used for the experiment.

Young plants of native species were tested; spear grass, *Aciphylla squarrosa* (Apiaceae), red tussock, *Chionochloa rubra* (Poaceae), Banks Peninsula blue tussock, *Festuca actae* (Poaceae), New Zealand flax, *Phormium tenax* (Asphodelaceae), and silver tussock, *Poa cita* (Poaceae) were purchased from Plantlife Propagators Ltd (Ashhurst, New Zealand). Seedlings of the exotic ryegrass (nil endophyte), a hybrid of *Lolium perenne* × *Lolium multiflorum* (Poaceae), were obtained from AgResearch Ltd (Lincoln, New Zealand). Mixed grass species comprising fescue, ryegrass and lawn grasses were sourced from a lawn at Lincoln University. Each plant was carefully transferred from its original pot to a 200 ml pot of 3–4 month-old potting mix comprising 80% bark, 20%, Osmocote Exact fertilizer (16-3.5-10), horticultural lime, hydroflo, pumice and allowed to grow in a glass house for two months before use in experiments.

2.3.2 Larval development and survival of porina on native and exotic hosts

For the no-choice bioassay, the surviving porina larvae (*n* = 53) were weighed, the potting mix changed and the larvae arranged in descending order by weight. Larvae were allocated randomly to one of the seven treatments: *A. squarrosa, C. rubra, F. actae, P. tenax, P. cita, L. perenne* × *L. multiflorum* and mixed grass spp. (*n* = 8 for all food sources except the mixed grass treatment, *n* = 5). Containers with larvae were placed randomly within the CT cabinet. Larvae were fed *ad libitum* with fresh foliage of the selected host plant every 3–4 days for six months, checked daily for mortality, and weighed monthly with the potting mix replaced in each container at weighing.

To determine the growth rate of the porina larvae on each host, the relative growth rate (RGR) was calculated and measured in grams of tissue gained per gram of caterpillar per day (Waldbauer, 1968).

$$\mathrm{RGR} = \frac{larval\ weight\ gain\ during\ feeding\ period}{(mean\ larval\ weight\ during\ feeding\ trial) * (duration\ of\ feeding\ trial)}$$

The effect of host diet on growth data (i.e. monthly weight gain of the larvae from initial weight) were analysed by ANOVA, while larval survival was evaluated using survival analysis. Statistical tests were conducted with GenStat® 18 and SigmaPlot 13.0.

2.4 Results and discussion

Larvae grew rapidly on the foliage of *F. actae*, *A. squarrosa* and *L. perenne* × *L. multiflorum*, but grew more slowly on *P. cita*, *C. rubra*, *P. tenax*, and mixed grass spp. A net increase in percentage larval weight was observed for all seven treatments after four months of feeding (Figure 2.1). The highest cumulative weight increases were observed for *F. actae* and *A. squarrosa* at 225% and 222% respectively, while mixed grass species had the least with 57%.

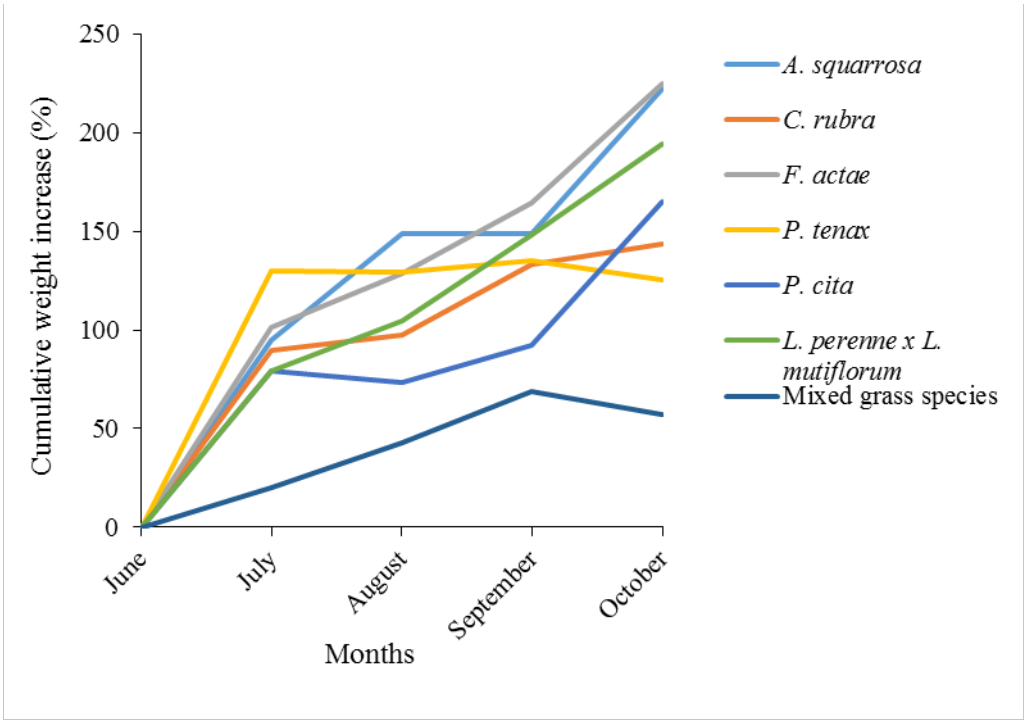

Figure 2.1 Effect of diet on weight increase of porina larvae.

The largest average weight gain of 284 mg was on *L. perenne* × *L. multiflorum*, an exotic host, while the smallest average weight gain of 28.8 mg was on *P. tenax* (Table 1). There was a significant difference in weight gain between larvae fed with *L. perenne* × *L. multiflorum* and *P. tenax* (F (6, 18) = 3.46, p = 0.019). The difference in weight gain observed between ryegrass and flax in this study may be due to the relative toughness of flax leaves. Leaf toughness appears to be an essential defence mechanism against another lepidopteran the European corn borer on maize, with tougher leaves suffering less feeding damage (Bergvinson *et al*. 1994). In a review of neonate lepidopteran ability to establish themselves on host plants, Zalucki *et al*. (2002) concluded that their success is dependent on both external plant factors (e.g. plant architecture, leaf hairs and trichomes, leaf toughness and hardness, leaf micro-flora and microclimate) and internal plant factors (e.g. nutrition, chemical defences, sequestered metals, constitutive defence expression, induced defence expression, resins and latex, and changes in host quality and interactions) that also play significant roles.

Mortality was lowest on ryegrass (13%), with larvae surviving the longest and on average 177 days. The highest percentage mortality of 75% was recorded on *A. squarrosa*, *F. actae* and *P. cita*, which also recorded the lowest average survival time of < 96 days (Table 2.1); however, these differences were not statistically significant (F (6, 26) = 9.25, p = 0.160). A few larvae survived to pupation, but only from ryegrass (2 pupae), *C. rubra* (1 pupa), *P. tenax* (1 pupa) and mixed grass spp. (2 pupae). One adult moth emerged from ryegrass and one from mixed grass spp. The mortality observed on larvae fed on the native hosts could be as a result of the nutritional quality and plant defences of these native hosts.

This study shows an inverse relationship between percentage mortality and average larval survival time.

Table 2.1: Effects of host plant on the weight gain, mortality and survival of porina

Plant species	Initial weight (mg):		*n*	Mean weight		%	Mean larval
				gain (mg)	std error	mortality	survival time (days)
	Mean	range					
L. perenne × *L. multiflorum*	414.6	298.0-551.4	8	284.0 [a]	40	12.5	177 [a]
A. squarrosa	427.4	334.6-635.0	8	91.0 [ab]	82	75.0	96 [a]
C. rubra	541.2	372.3-751.1	8	117.0 [ab]	39	37.5	161 [a]
F. actae	452.1	308.7-617.3	8	197.0 [ab]	115	75.0	96 [a]
P. tenax	563.6	389.9-971.0	8	28.8 [b]	42	37.5	154 [a]
P. cita	454.4	327.5-716.5	8	95.0 [ab]	78	75.0	77 [a]
Mixed grass spp.	413.2	327.6-524.5	5	150.0 [ab]	26	60.0	112 [a]

* Weight gain = final weight - initial weight

The lowest relative growth rate of 0.0057 (gm/gm larva/day) was recorded on larvae fed with mixed grass spp., while the highest was on *F. actae* although this was not significantly different from the RGR for *A. squarrosa* and *L. perenne* × *L. multiflorum* (Figure 2). The larvae grew the fastest on *F. actae*, *A. squarrosa* and *L. perenne* × *L. multiflorum* and slowest on mixed grass spp.

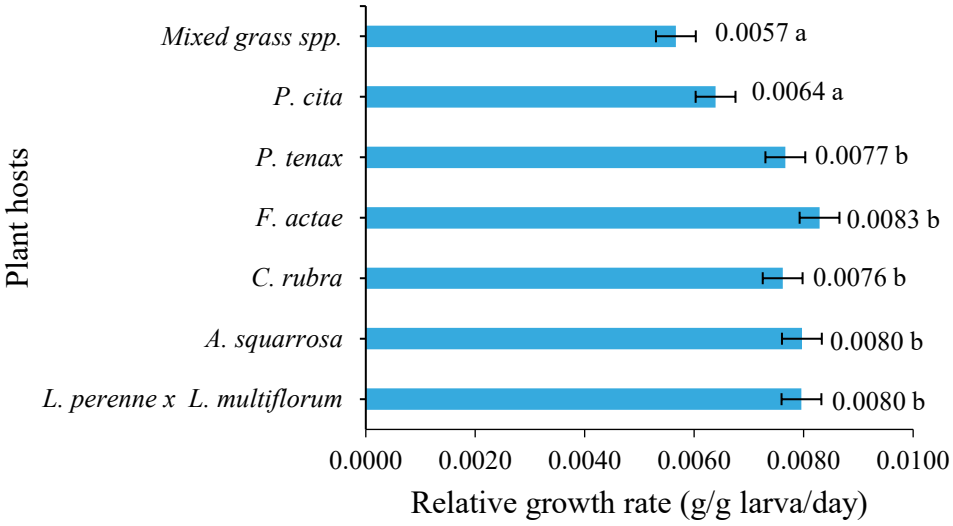

Figure 2.2: Mean relative growth rate of porina larva (g/g caterpillar/day) on native and exotic host plants. Means followed by the same letter are not significantly different (P > 0.05).

2.5 Conclusions and future work

This study was constrained by the variation in the age of the larvae used and the sample size. However, the results clearly show that porina larvae can feed and develop on some of the native host plant species tested. A further study using a larger sample size and larvae of uniform age from several porina species is being conducted to validate this result. Additionally, the nutritional quality of the host plants is being analysed to determine the influence of primary metabolites on porina larval development on these hosts.

Chapter 3

Seasonal flight and identification of *Wiseana* species in Canterbury pastures and implications for their management

3.1 Abstract

Effective management of porina in New Zealand pastures relies not only on pasture age, history and management, but also on understanding which *Wiseana* species are present and the seasonal dynamics of their flight within a given geographical area. Weekly light trap catches over two porina flight seasons were monitored and, using molecular methods of identification, the results showed that *W. cervinata* and *W. copularis* were the two main species infesting pastures in Canterbury. The conclusion was that *W. cervinata* flew from late spring to early summer and *W. copularis* from early summer to late summer. This study describes how data from monitoring a simple light trap can be used to determine when to apply the most effective control strategy.

Key words: Flight, peak, porina, *Wiseana cervinata*, *Wiseana copularis*

3.2 Introduction

Insect dispersal and migration are phenomena that have held the attention of both laypeople and scientific thinkers from ancient times and their detailed study dates back to the early years of natural history (White 1789). Dispersal refers to the scattering of a population and increasing the mean distance between individuals (Schneider 1962, Southwood 1981). This involves both migratory and non-migratory movements (Danthanarayana 1986). Non-migratory movement's concern travel within the habitat associated with such activities as feeding, mating, and oviposition (Danthanarayana 1986). Migration, on the other hand, takes insects beyond their natal habitat to colonize new habitats, re-colonise old ones and to locate aestivation and hibernation sites. (Johnson 1969, Southwood 1962, Dingle 1972). Essentially it is an evolved adaptation for survival and reproduction (Johnson 1960). Studies such as the heteroecious movement of aphids between two host plants (Johnson 1956, Schneider 1962, Dixon & Howard 1986), the pioneering study of honeybee foraging flight to and from their hive (van Frisch 1967), and movement of whiteflies over short distances (Byrne 1999) have driven research on insect dispersal. In contrst, the impressive mass movement of butterflies and dragonflies (Williams, 1930, 1958), and the locust plagues of the 1940s and 1950s have have focused on insect migration (Kennedy 1951, Rainey 1989). Insects modulate their flight activity in response to environmental cues (Drake & Reynolds 2012) through

behavioural adaptations in relation to food availability (Chapman & Drake 2010). Accordingly, the geographic distribution and population dynamics of insect species, including the diel periodicity of individuals, are strongly influenced by abiotic factors, such as temperature, photoperiod, wind speed and rainfall (Pellegrino *et al*. 2013).

Some authors, such as Janzen & Schoener (1986) and Tanaka & Tanaka (1982) have suggested that peak populations of many insect species are often associated with suitable rainfall, or moisture conditions, which can increase food availability, provided temperatures are suitable. However, Kasperi (2001) was of the opinion that fluctuations in species population densities could be explained only partially by moisture availability or even not related to increasing rainfall at all. As a result, Didham & Springate (2003) opine that although moisture might be a cue for life-history development, other factors such as temperature or resource availability may drive variability in growth, survival, reproduction and mortality rates that ultimately affect population dynamics. Wind may also play a role in movement across short and long distances (Chapman *et al*. 2011), as well as in emergence from pupation (Helson & Penman 1970) and the emission of, and response to, sex pheromones during mating (McNeil 1991). Consequently, short-term changes in weather can influence insect flight behaviour (Henson 1951, Wellington 1957). Therefore, since insect flight behaviour is dynamic, it should be viewed in relation to the meteorological systems instead of being based on specific, static data at a given point in time (Henson & Penman 1980).

The flight pattern of porina, has been considered very variable among species within the *Wiseana* complex, as well as between districts and over time (Carpenter & Wyeth 1980, Barlow *et al*. 1986, Brown *et al*. 1999). However, the mechanism that triggers flight is yet to be fully understood. Cottier (1962) observed that flights occurred on calm night or nights with a slight breeze with temperature above 8.3℃ after a warm day. Helson (1967) stated that flights occur on both cloudy, or clear cool nights and have been observed during periods of misty rain or just prior to rain. Dumbleton (1945) was of the opinion that porina flight is governed by temperature, since its development from egg to adult and the emergence of adult moths is driven by soil temperature. Dick (1945) also considered that both development and emergence of adults are driven mainly by soil temperature, with eclosion taking place on days with high soil temperature. However, Pottinger (1968) later suggested that other factors such as light or wider synoptic events such as frontal weather systems, could also be important. That study also suggested that dispersal flights have an important influence on the dynamics and infestation of pastures by porina, since he measured immigration and emigration of moths into and out of a paddock that was previously devoid of any

larval population. This was in contrast to Dick (1945) who had found no such indication of migration.

Much attention has been given to monitoring mating flights of porina because female moths are very fecund, laying over 3000 eggs (Ferguson et al. 2019). Evidence is strong that the amount of damage caused by larvae in winter is directly related to the intensity/size of adult flights during the previous spring and summer, and thus the volume of eggs laid (e.g. French 1979, Barratt *et al*. 1999). Consequently, peak flight has been used as a bio-fix by farmers for the timing of control strategies, such as general insecticide application (Weller 1968), mob stocking (French 1979) and, more recently, the insect growth regulator diflubenzuron (Ferguson *et al*. 2016). From the time of peak flight the time when large numbers of early larval instars of porina will be dwelling on the soil surface can be estimated. After hatching, young porina larvae live on the soil surface for 4-6 weeks and are vulnerable to pesticides before they burrow into the soil, from which they emerge only at night to feed (e.g. Dumbleton 1945, Dugdale 1994, Barratt *et al*. 1999, Ferguson & Crook 2004). This early period is targeted to improve control by diflubenzuron which prevents the larvae from moulting to the next instar. However, for death to occur, it must be applied when the larvae will moult shortly after application, and moulting is more frequent in the early developmental stages (Ferguson *et al*. 2019) when larvae are close to the soil surface. This in turn has caused a revival of interest in the seasonal variation of the life cycle of porina. Accordingly, optimal control requires an awareness of differences between porina species with regard to the timing of adult flight patterns, egg hatching and larval development (Ferguson & Crook 2004).

The objective of this study was to determine the species of *Wiseana* moths flying in Canterbury, when the flight begins, and each species' seasonal flight pattern. Such data can then be used to estimate when management strategies will be most effective.

3.3 Method

3.3.1 Insect Trapping

During the 2015-16 (7th December 2015- 4th April 2016) and 2016-17 (22nd August 2016-3rd April 2017) porina flight seasons, a light trap (Figure 1) located on the AgResearch farm (43°38'10.6"S, 172°28'19.5"E), Springs Road, Lincoln, Canterbury, was set to be on between 9:00 pm and 1:30 am and cleared once a week. Adult porina moths are attracted to the light of the trap and, as they flutter around the light, they slide down to the bottom and become trapped. The contents of the light trap were emptied into a plastic bag taken to the laboratory, sorted, and counted. Each moth

was put inside a glass vial containing 96% ethanol and stored inside a fridge at 4 °C for subsequent molecular identification (see 3.3.2).

During the flight seasons, live adult females were also caught by hand at the trap and put individually into numbered 120 mL plastic bottles to lay eggs. The eggs were maintained in maternal groups and reared at 15 °C using the method developed by Atijegbe *et al*. (2017, Chapter 4) to produce larvae for subsequent bioassays (Chapters 5, 7 and 8). The female parents were then stored in ethanol for molecular identification to species (section 3.2.2) so that their offspring could also be identified to the correct *Wiseana* species. The number of females caught by hand each week was added to the number trapped weekly to make up the total number trapped each week. The weather conditions during the trapping periods was obtained from the National Institute of Water and Atmospheric Research (NIWA) database (https://cliflo.niwa.co.nz/).

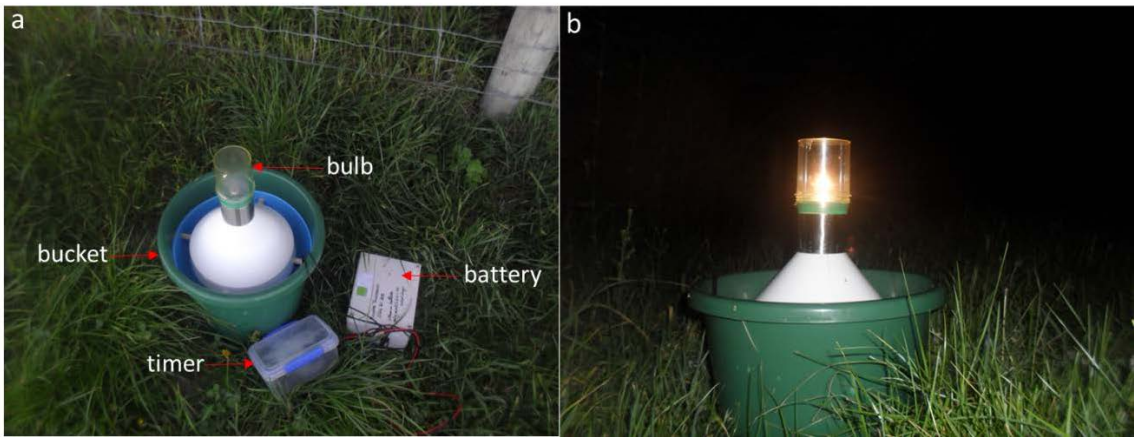

Figure 3.1: Light trap, (a) showing its components, and, (b) lit trap at night during trapping.

3.3.2 Molecular species identification

Identification of porina species during the 2015-16 flight season was made using the polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) method developed by Richards *et al*. (2017). This produces species-specific markers from the sequence of the cytochrome oxidase I (COI) DNA barcode region (Hebert 1995) using either a double restriction enzyme digest of the PCR amplicon with *Aci*I and *Bcc*I or single digest with *Dde*I. The trap catch for the 2016-17 flight season was identified using the same PCR-RFLP method as well as a high-resolution melt (HRM) method with primers LCO2355 and HCO2461, also developed by Richards *et al.* (2017). DNA was extracted from one leg of each moth using the Genomic DNA Mini Kit Tissue protocol (Geneaid™, New Taipei, Taiwan). Proteinase K digestion was then performed at 60°C for at least 30

min. To maximise DNA recovery, final elution was performed using two sequential washes of 100 µL elution buffer, yielding a final volume of 200 µL DNA. The DNA was then stored at 4-8℃ until used for PCR analysis.

3.4 Results

3.4.1 Molecular identification of *Wiseana* moths

Double restriction digest of the ~700 bp COI PCR fragment created unique diagnostic profiles for *W*. *copularis* (two haplotypes), *W*. *cervinata,* and *W. umbraculata* (Table 3.1, Figure 3.2). That for *W. umbraculata* was not cut by those enzymes but was by *Dde*I to produce a ~270 bp and ~430 bp compared to a ~90 bp and ~610 bp fragments for the other two species (Table 3.1). The more rapid HRM method produced a 106 bp amplicon and unique melt profiles for all species, with high-resolution melt temperature peaks of 73.3℃ for *W*. *copularis* (S[1] profile 1 and 2), 74.1℃ for *W*. *cervinata* and 74.7℃ for *W*. *umbraculata* (Figure 3.3).

A total of 263 moths were caught during the 2015-16 season of which 151 (57%) were identified to species according to their molecular profile compared to that of known species standards (Figure 3.2). Of these, 129 were identified as *W. copularis* (S profile 1 and 2, Table 3.1), 20 as *W*. *cervinata*, and two as *W. umbraculata*. During the 2016-17 flight season, 510 moths were caught with 256 (50%) identified to species, being 170 as *W. copularis* (S profile 1 and 2), 82 *W*. *cervinata*, and four of *W. umbraculata* (Figures 3.2, 3.3).

Table 3.1 COI PCR-RFLP haplotypes for three porina species.

	Size of the fragment (bp)	
Species	***Aci*I and *Bcc*I**	***Dde*I**
W. copularis - S profile 1	40, 90, 120, 150, 300	90, 610
W. copularis - S profile 2	90, 120, 190, 300	90, 610
W. cervinata	180, 210, 310	90, 610
W. umbraculata	700 (no sites)	270, 430

S - Southern haplotype

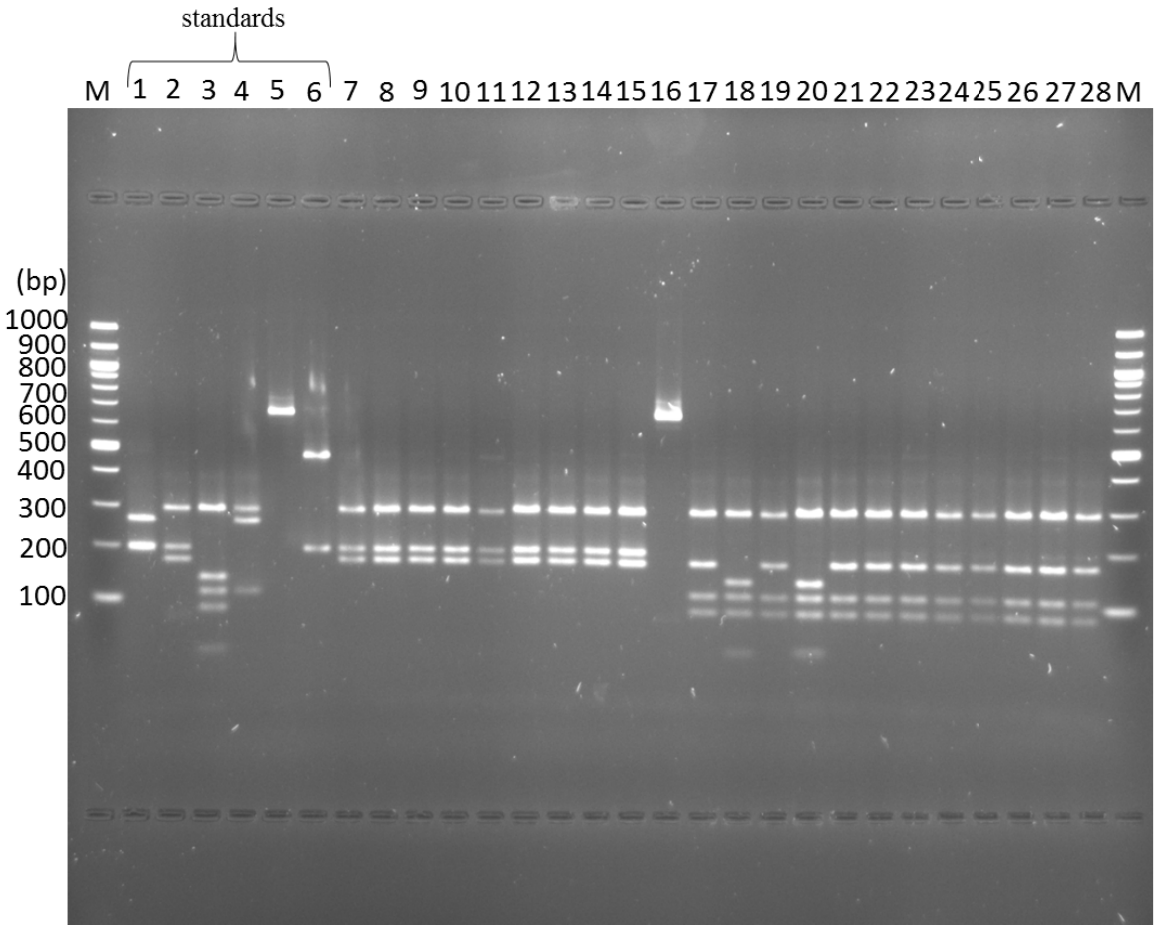

Figure 3.2: COI PCR-RFLP profiles of porina after double digest with the *AciI* and *BccI*. M - 100 bp DNA marker, 1 - *W. signata*, 2 - *W. cervinata*, 3 - *W. copularis* (S profile 1), 4 - *W. copularis* (northern haplotype), 5 - *W. umbraculata*, 6 - *W. fuliginea*; 7-15, *W. cervinata*; 16, *W. umbraculata*; 17-28, *W. copularis* (S profile 1) and 18 & 20 - *W. copularis* (profile 2).

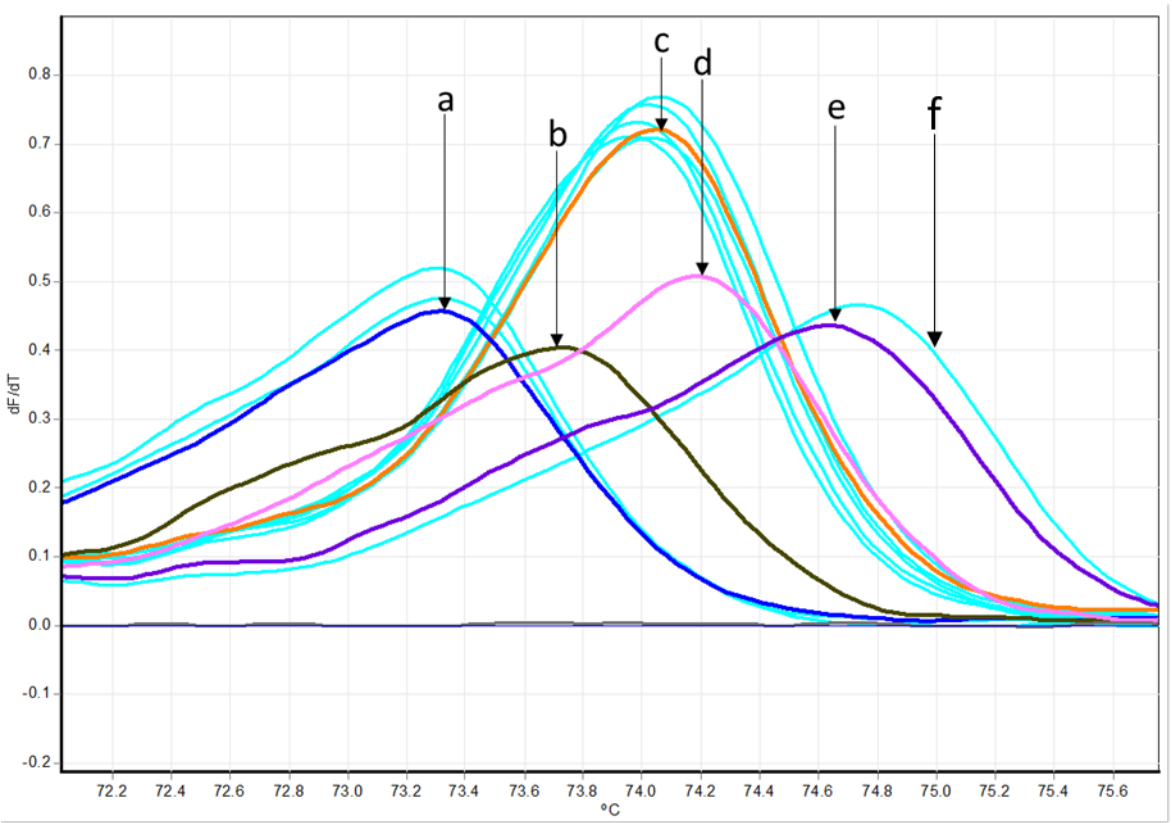

Figure 3.3: HRM peak profiles for *Wiseana* species after COI PCR amplicons have been denatured illustrated for (a) *W. copularis* standard (profile 1 & 2), (b) *W. jocosa* & *W. mimica* standard, (c) *W. cervinata* standard, (d) *W. fuliginea* standard, (e) *W. umbraculata* standard, (f) specimens .

3.4.2 *Wiseana* flights

The light trap for the 2015-2016 season was set up late in porina flight season, due to a combination of delays obtaining the trap and securing the best location to place it. Trapping initially targeted collection of only adult females for their eggs, which were later to be hatched (Chapter 4) and reared to obtain the larvae and pupae for subsequent bioassays (Chapters 5, 7 and 8). Before weekly trapping began, a total of 24 females were caught by hand between the 15th of October and the 11th of November 2015, with the prevailing weather condition of the night during flight recorded. Using the COI PCR-RFLP described above, they were identified to species and the result presented in Table 3.2.

Table 3.2: Female moths caught before weekly trapping commenced.

Dates	Number	Species	Weather conditions
16-Oct-2015	2	*W. cervinata*	clear sky, windy
18-Oct-2015	1	*W. umbraculata*	cloudy, drizzle, slight wind
19-Oct-2015	2	*W. cervinata*	clear sky, slight wind
21-Oct-2015	4	*W. cervinata*	clear sky, slight wind
24-Oct-2015	3	*W. cervinata*	clear sky, windy
30-Oct-2015	1	*W. umbraculata*	full moon, cloudy, no wind
1-Nov-2015	7	*W. cervinata*	clear sky, slight wind
5-Nov-2015	2	*W. cervinata*	clear sky, slight wind
11-Nov-2015	2	*W. copularis*	full moon. slight wind

Weekly trapping for the 2015-16 flight season commenced on the 7th December and ended on the 14th of March 2016. During this 14-week trapping period, the species *W*. *copularis* (profiles 1 and 2) was identified to be flying with a single flight peak that occurred on the 18th of January (Figure 3.4a). The weather conditions using the NIWA database during this period showed that average weekly air temperature was 15℃, soil temperature at a depth of 10 cm was 18℃, wind speed of 16 km/h close to the soil surface, and rainfall of 2 mm. During this period, 62 female moths caught for egg collection were identified as two individuals of *W*. *umbraculata*, 16 of *W*. *cervinata*, and 44 of *W. copularis*.

In the 2016-17 season the light trap was set-up in the field much earlier than the 2015-16 season, before porina flight had started. Porina flight commenced on the 3rd of October 2016 and trapping commenced on the 10th of October 2016 and ended on the 12th of March 2017, comprising a 23-week trapping period (Figure 3.4b). *Wiseana cervinata* commenced flight the 1st week of October through to the 1st week of November, comprising a short 5-week duration with a distinct peak in numbers of porina flying recorded for the week ending on the 31st October 2016 (peak 1, Figure 3.4a). The NIWA weather conditions during this period showed that average weekly air temperature was 13℃, soil temperature at a depth of 10 cm was 14℃, wind speed of 13 km/hr close to the soil surface, and rainfall of about 3 mm. This was closely followed by the first flight of *W*. *copularis* occurring in the 2nd week of November 2016 and continuing to the 16th January 2017. Two distinct flight peaks were recorded, a lower peak occurring during the week ending 5th

December 2016 (peak 2, Figure 3.4b) and a higher peak during the week ending 9th January 2017 (peak 3, Figure 3.4b) of a 10-week flight period. The weather conditions during this period indicated that average weekly air temperature was 15℃, soil temperature at a depth of 10 cm was 18℃, wind speed of 17 km/h close to the soil surface, and rainfall of about 2 mm. A second late, much smaller and shorter 2-week flight period of *W*. *copularis* occurred from the 2nd week to the 4th week of February 2017 (4, Figure 3.4b). Four *W. umbraculata* moths were caught during the entire 2016-17 flight season, one was caught at the time *W. cervinata* were flying and the remaining three trapped when *W. copularis* were flying. Also during the 2016-17 flight season, the 100 females collected for their eggs were identified as one individual *W. umbraculata*, 10 *W*. *cervinata* and 89 *W*. *copularis*.

Although weekly trapping started late during the 2015-16 season, the flights of *W*. *cervinata* during both seasons started early in October and ended in early November (Table 3.2, Figure 3.4a and 3.4b) and were short in duration. Similarly, flights of *W*. *copularis* during both seasons started early in November and ended in early March, and were much longer in duration. Peak flight of *W*. *copularis* in both seasons seemed to occur around the same time, the week ending 18th of January during the 2015-16 season and the week ending 16th of January during the 2016-17 season. However, while flights of *W*. *copularis* declined gradually from a peak during the 2015-16 season until flights ended in March, in the 2016-17 season there was an abrupt cessation of *W*. *copularis* flight during the five-week period from its peak (peak 3, Figure 3.4b) on 16th January to the 13th of February 2017. The weather conditions during this period showed that the average weekly air temperature was 17℃, soil temperature at depths of 10 cm was 20℃, wind speeds of about 16 km/h close to the soil surface, and rainfall of 0.7 mm. Correlation analysis between flight times and weather during the 2015-16 season showed strong correlations for air temperature (r = 0.77) and soil temperature (r = 0.60), and a moderate to weak correlation for wind speed (r = 0.32) and (r = 0.27) rainfall respectively. The 2016-17 season also recorded strong correlations for air temperature (r = 0.84) and soil temperature(r = 0.73), while that for rainfall (r = 0.19) and wind speed (r = 0.03) were very weak.

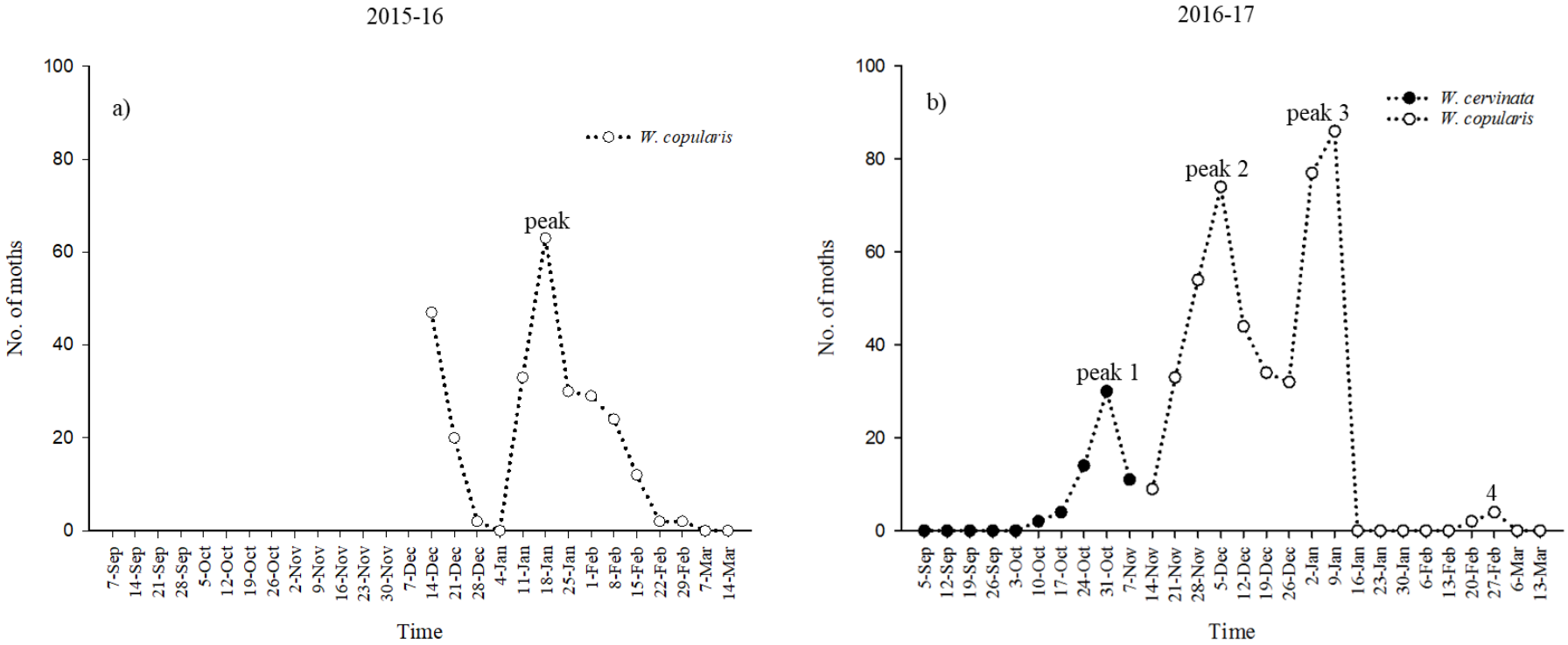

Figure 3.4: Number of moths trapped weekly during, a) the 2015-16 flight season, and, b) the 2016-17 flight season.

3.5 Discussion

The use of PCR-RFLP and HRM methods developed by Richards *et al*. (2017) for species identification enabled the real-time assessment of which species were flying when in Lincoln, Canterbury. This approach allowed for 50-100 samples to be quickly processed at one time and cost-effectively (at a cost of < $7 per sample) to overcome the unwieldy, time consuming and expensive constraints of the methods of MacArthur (1986), Herbert (1994) and Brown *et al*. (2000). As a result, *Wiseana cervinata* and *W*. *copularis* (S profiles 1 and 2) were the main species found during both 2015-16 and 2016-17 flight seasons, with *W*. *copularis* the more common of the two. However, a few adults of *W*. *umbraculata* were caught during the flights of *W. cervinata* in spring and *W*. *copularis* in summer which suggests that the flight of *W*. *umbraculata* may not distinct, but rather this species flies in low numbers over a long period when *W. cervinata* and *W*. *copularis* are also flying. Recently Richards *et al*. (2017) reported that *Wiseana cervinata*, *W*. *umbraculata* and the two southern haplotypes profiles of *W*. *copularis* are the species found in Canterbury, which is consistent with the findings of this study.

The timing of flights by *W. cervinata* and *W. copularis* were synchronised and distinct in both seasons, with *W. cervinata* commencing flight earlier than *W. copularis* as indicated previously by Helson (1967) and French (1973). The variation in their flight times suggests a difference in how quickly these species can complete development. Both air and soil temperature appear to play a key role in this as suggested by the strong correlations with these factors during both flight seasons. Supporting evidence for this is also provided by the study of Ferguson & Crook (2004) on the larval development of the two species which revealed that *W. cervinata* developed faster than *W*. *copularis* over a range of temperatures. The same holds for the time it takes for the eggs of both species to hatch (Ferguson & Crook 2004, Atijegbe *et al*. 2017). However, the basis for why the flight period for *W. cervinata* is short compared to that of *W*. *copularis* is still not clear. One potential reason could be a difference in abilities to develop at low temperatures. Accordingly, with the mean air and soil temperatures of 13℃ and 14℃, respectively, this suggests that *W. cervinata* are able to complete development at lower temperatures (psychrophilic) and emerge in early to mid of spring. In contrast, the higher mean air temperature of 15℃ and soil temperature of 18℃ indicates that *W*. *copularis* develop best at moderate temperature (mesophilic), to emerge in late spring to the end of summer when conditions are warmer.

The variation in flight pattern and corresponding flight peaks between seasons, coupled with weather conditions observed on the nights moths were flying, suggests that local weather conditions determine when the moths fly (Hurst *et al*. 2019). However, it seems that actual eclosion of adult moths may not happen immediately once pupation is completed. Rather the moths may wait until local weather conditions are suitable for flight and begin to emerge as soon as conditions start to improve. The consequence is mass emergence with defined peak flights. For *W*. *cervinata* such ideal weather

conditions appear to be characterised by a mean wind speed of ~13 km/h, air temperature of ~15℃, soil temperature at a depth of 10 cm of about ~14℃, and rainfall of ~3 mm. That for *W*. *copularis* is wind speed of ~17 km/h, air temperature of ~15℃, soil temperature of ~18℃, and rainfall of ~2 mm. In the same way, extended periods of bad weather such as rain, frost or dry spells, can prevent emergence and flight. This is observed when *W*. *copularis* was flying in the weeks between the 16th of January and 13th of February 2017 (Figure 3.4b), when average air and soil temperatures were 19℃ and 20℃ respectively. This result supports the hypothesis postulated by Helson & Penman (1970), that the mechanism triggering mass emergences of porina is a rise in soil temperature above 10℃ to a depth of 10 cm in the spring.

In the 2016-17 flight season, *W. cervinata* had a single peak while *W. copularis* had a bimodal peak, and each peak likely represented a different age cohort of porina offspring. This implies that these species, with three different cohorts, probably caused the reported damage on pastures from autumn to winter of 2017 in Canterbury (Morris *et al*. 2016). The current practice recommended for porina by AgPest™ (http://agpest.co.nz/) is to track flight numbers during the season and use this information to determine when to apply diflubenzuron, which is most effective on the early larval instars of porina. Timing is even more important as diflubenzuron degrades rapidly after application with a half-life of less than seven days (Tomlin 1994), so the vulnerable larval stages have to be at the soil surface within that short window of time.

During a flight season it is usual to have multiple flights from one or more porina species, which in some cases may be month's apart (Barratt *et al*. 1999). This creates bimodal or polymodal peaks in flight activity and leads to overlapping age cohorts of larvae within and among porina species in pastures. The difference in phenologies and peak flight times of *W. cervinata* and *W. copularis* have implications for the control of porina in pasture, because the larvae of *W. cervinata* moult frequently and are susceptible to control by diflubenzuron before *W. copularis* eggs have hatched. Later, *W. copularis* larvae will be at a similar susceptible stage when those of *W. cervinata* may have almost completed moulting and be much less vulnerable (Ferguson & Crook 2004). Understanding these differences in their development and quantifying their presence allows for better-targeted control of the two species. For example, during the 2016-17 season larvae from the early *W. cervinata* flight (peak 1) would hatch from eggs laid on the 21st of November 2016 and be approximately 9-weeks old on about the 21st - 25th of January 2017 (using the 10 -12 weeks vulnerable window - 3 weeks for eggs to hatch and 9 weeks after hatching, larvae are still on the soil surface and vulnerable). On the other hand, larvae from the first *W. copularis* flight (peak 2) would hatch between the 7th -11th of January 2017 and be approximately 3-weeks old about the 21st - 25th of January 2017. Both species would still likely be susceptible to treatment between the 23rd and 30th of January, because both cohorts would be under the 10-12 week window identified as the period over which the first three larval instars are

developing and vulnerable. In the same vein, larvae of *W. copularis* from the second flight (peak 3) would hatch from the 4th - 8th of February 2017 and be 9-weeks old on about the 8th - 12th of April. Equally larvae from the small, late flight of *W*. *copularis* (4 in Figure 3.4b) would have hatched between the 25th - 29th of March and be 1-week old about the 1st - 5th of April, therefore still vulnerable to diflubenzuron between the 10th and 17th of April. So for 2016-2017 season at Lincoln, the most cost-effective control strategy would have been two targeted applications of diflubenzuron, the first applied between the 23rd and 30th of January and the second between 10th and 17th of April (Ferguson & Cook 2004).

Regional differences in species composition, local weather conditions and flight times typically impact porina control. For example, in 2016 in the Manawatu, North Island, where *W. copularis*-northern haplotype and *W. signata*, the main species are flying from October to March, diflubenzuron application was recommended in June later than was usually applied. This was to reflect a late and large flight of *W*. *copularis* in late March (Ferguson *et al*. 2016). Also on the West Coast of the South Island, *W. copularis* is the main species and flights usually begin in October through to March with diflubenzuron applied from late February to early or mid-March (Mansfield *et al*. 2017). The information on species composition, the time flight commenced together with the local weather condition are essential for the effective management of *Wiseana* using diflubenzuron in a given location. This adds diflubenzuron in a pest management strategy as a popular choice by farmers because of its low cost of about $25–90/ha for ground spraying to helicopter spraying (Askin & Askin 2014) and its relative safety for users and many non-target insects alike. Moreover, it leaves sufficient larvae in pastures (Ferguson *et al*. 1996) to allow the persistence of naturally occurring pathogens that subsequently suppress any residual populations (Crawford & Kalmakoff 1977).

In conclusion, this study has shown that *W. cervinata* and *W. copularis* (S profile 1 and 2) are the two main species that fly in Canterbury. Light traps are a key tool for farmers for monitoring porina flight patterns and larval infestations in New Zealand pastures. The identification of peak flights in a given area during a flight season can be used by farmers and their advisors to time porina control effectively. Finally, it is possible to use seasonal flight and weather data of a location in New Zealand, together with current knowledge on porina development, to develop a simple model that can be used to predict more accurately the best time to control porina (Chapter 9 and 10).

Chapter 4

Laboratory handling and rearing of early instar porina

Results of this chapter was published as:

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4.1 Abstract

Mass rearing *Wiseana* (porina) species, a major endemic insect pest in New Zealand pastures, has proved challenging for research and pest management and has presented problems over several decades in the laboratory. Researchers have acknowledged that handling the eggs and first instar larvae are of critical importance during laboratory rearing. A simple method is presented that improves survival of early instar porina larvae. Eggs of three porina species (*W. cervinata, W. copularis, W. umbraculata*) hatched faster at 22℃ than at 15℃.

Key words: *Wiseana*, rearing methods, temperature, development time

4.2 Introduction

Wiseana Viette (Lepidoptera: Hepialidae), also known as porina, is a complex of seven species endemic to New Zealand, two of which are major insect pests of pastures that are critical for livestock production in New Zealand. With the reclassification of this species complex (Dugdale 1994), it is essential to correctly identify species in pasture in different parts of New Zealand and to be able to rear them for research purposes. The greatest challenge in conducting research with porina is the difficulty in laboratory rearing because of their long life cycle of 12-14 months. Allan *et al*. (2002) reported that porina larvae could be difficult to rear in the laboratory for research purposes from eggs. As a result, very little is known about the fundamental biology of porina, their native habitat(s) and what porina feed on in these habitats. In pastures, porina eggs and first instar larvae are very susceptible to desiccation (Stewart 2001), and dry summers during the egg and early instar stages usually result in small porina numbers the following winter (Ferguson CM, personal communication). Newly hatched porina larvae are much more susceptible to desiccation than eggs, and the susceptibility of *Wiseana* spp. larvae to desiccation decrease with age (Stewart 2001). The most drought-susceptible stage in porina's life cycle is the egg during hatching, and hatching does not occur at 0% relative humidity (RH) (Stewart 2001). The rate of development of porina eggs is significantly affected by temperature (French & Pearson 1979), as is the case for all insects. Although porina lay a

large number of eggs, mortality of early instar larvae is very high (Barratt *et al*. 1990), and even in late instars can be as high as 90% (Moore 1972, Fleming *et al*. 1986). In the laboratory, current challenges in rearing porina include larvae drowning after hatching in the container, contamination from disease-causing organisms (bacteria, fungi and viruses) on the egg surfaces, diseases from viruses transmitted from the mother to her offspring, competition/cannibalism of larvae in rearing containers, the composition of the medium that larvae are transferred onto after hatching for rearing, and the type of diet fed to larvae during rearing (Allen *et al*. 2002). The rearing from eggs to adults in the laboratory provides opportunities to study the physiology of the immature life stages and how to manage them. It also makes species identification of larvae using DNA easier and cheaper because only the parent needs to be identified, compared to field collected larvae where every individual needs to be DNA tested.

Various studies have previously attempted to rear hepialid larvae in the laboratory, but with limited success. Waller (1968) unsuccessfully tried to rear porina larvae in peat-filled cans, but achieved some success rearing larvae to first instar in agar-filled dishes. Elder (1970) reared the hepialids *Oncopera brachyphylla* and *O*. *mitocera* in a multi-temperature incubator ranging from 10–27°C, and found that the optimal rearing temperature was dependent on species. The most successful method reported to date was that of Wood (1970), who reared porina larvae in 5-inch plastic flower pots nearly filled with a mixture of two parts of sterilised earth, one part of old pine sawdust to which 1 to 16 parts of fine sand was added. They were fed wilted clover, and 37% of larvae emerged as adults. Dodgshun (1970) studied viral diseases of *Wiseana* spp. and found that use of an artificial maintenance medium saved much time when feeding larvae and adapted the semi-synthetic maintenance diet first employed by Shorey & Hale (1965) to raise noctuid larvae. French & Thomas (1971) reared field-collected larvae on agar for 100 days by feeding them clover leaves. French & Pearson (1979) studied the effect of temperature at 100% relative humidity (RH) on the rate of porina egg development under different constant temperature regimes and found that the number of days required for hatching increased with decreasing temperature. Wagner (1989) used carrot to rear larvae of the hepialid moths *Hepialus humuli*, *H. californicus*, *H. behrensii*, *H. hectotoides*, *Phassus triangularis* and *Korscheltellus gracilis* to adults. Carpenter (1983) showed that copper oxychloride enhanced the viability of porina eggs in laboratory cultures. It overcame mould problems associated with laboratory cultures of porina eggs, and is cheap and easily obtained. Stewart (2001) reported that newly hatched larvae of the pasture pest *W. copularis* were much more susceptible to desiccation than eggs, except for those in the process of hatching. There was no successful hatching at 0% RH. Ferguson & Crook (2004) reported that egg hatch time decreases with an increase in temperature from 10–20℃ for *W. cervinata* and *W. copularis*. Popay (2001) reported a mass-rearing technique for porina adults from eggs using a diet with seven ingredients including clover and carrot that was modified from the semi-synthetic diet of Dodgshun

(1970), but no data on survival rate were provided. Using this method, larvae were reared through to adults in about eight months. Ferguson & Crook (2004) successfully reared larvae hatched from eggs collected from adults that emerged from field populations. The larvae were reared individually at 10, 13, 16, and 20℃ in Petri dishes containing moistened bark and fed a semi-synthetic diet based on white clover and carrot with 61*% W. cervinata* and 68*% W. copularis* larvae pupating successfully at 16℃. Similar success was also achieved when the artificial diet was substituted with white clover, without formaldehyde at the second or later larval instars, and with a commercial potting mix (Yates Thrive) instead of bark (Ferguson unpublished). The method currently used by researchers for rearing porina is based on the methods of Popay (2001) and Ferguson & Crook (2004). It involves sterilisation by washing eggs in a solution of 1 mg/litre of copper sulphate, rinsing them with distilled water and then transferring them to damp filter paper in Petri dishes and keeping them at 20℃ to hatch.

This study presents modifications to current rearing methods that are particularly useful for experimental studies of host-plant responses in porina.

4.3 Materials and methods

4.3.1 Egg collection and surface sterilisation

Adult female porina moths were hand collected between 21:00 h and 01:30 h from a light trap located at the AgResearch farm (43°38'10.6"S 172°28'19.5"E) on Springs Road, Lincoln. Individual females were put into numbered 120 mL plastic bottles so that the larvae from each female could be matched back to their parent after each adult was identified to species using the DNA method of Richards *et al*. (2017). Moths were placed at room temperature close to a light source for about 4 hours, and they were excited by shaking the container once or twice to make them lay their eggs within 1-2 days.

Filter papers (90 mm) were immersed in a solution of 1 mg/litre of copper sulphate for 2 minutes and were drained on paper towels to remove excess solution. Moist filters papers were placed at the bottom of Petri dishes (90-mm diameter) and 50 eggs/Petri dish from each female were spread thinly over the papers to sterilise the eggs. The Petri dishes (n=30) were covered with their lids and sealed on the side with Parafilm® to retain moisture and to prevent larvae from escaping. Ten Petri dishes per species were prepared, containing eggs of W. *copularis, W. cervinata* and *W. umbraculata*, respectively. Petri dishes for each species were split into two batches; one batch was kept in a constant temperature (CT) cabinet (CAT.190 RHS, Contherm Scientific Ltd) at 15℃ and remaining Petri dishes were stored in a second CT cabinet at 22℃. The photoperiod in both cabinets was with 12L: 12D. The eggs were observed daily until eggs started to hatch and the date recorded.

4.3.2 Rearing medium and larval diet

Once larvae started hatching, all 50 eggs/larvae from each Petri dish were transferred immediately into a 3 L Sistema® plastic container (230 mm x 175 mm x 120 mm) half filled with Grade-2 moist horticultural bark (HB) sourced from Intelligro (formerly known as Southern Horticultural Products). There was a separate container for all the larvae from each Petri dish. The larvae were fed weekly with carrot (*Daucus carota*) pieces for 8 weeks. The epidermal layer was scraped off the carrot and it was dipped in hot water for 1 min to remove any residual pesticides then chopped with a knife into pieces about 50–100 mm thick.

4.3.3 Statistical analysis

One-way ANOVA was used to compare hatching times between porina species at each temperature. Post-hoc comparison using Student–Newman–Keuls (SNK) was done to separate means.

4.4 Results and discussion

Early attempts at rearing the eggs using the washing technique described by Popay (2001) often resulted in drowning of newly hatched larvae (up to 90% mortality) due to excess moisture (data not shown). The modified sterilisation technique described here reduced moisture levels so that larval survival improved substantially (Table 4.1). Both soil and vermiculite were tested initially but these needed to be moistened once or twice a week to avoid desiccation of larvae whereas Grade-2 moist bark retained moisture and needed changing only once per month.

Table 4.1: Hatch time (mean ± sd) for eggs and survival (mean ± sd) at 8 weeks for larvae of three porina species (*Wiseana* spp.) at two rearing temperatures. Means in the same column followed by the same letter are not significantly different (P>0.05).

	15℃			**22**℃	
Porina species	**Hatch time**	**N**	**No. survived**	**Hatch time**	**N**	**No. survived**
W. cervinata	16 ± 1.7[a]	50	47 ± 1.0[a]	9 ± 1.6[a]	50	45 ± 1.9[a]
W. copularis	32 ± 15.0[b]	50	45 ± 1.6[a]	17 ± 1.9[b]	50	46 ± 1.6[a]
W. umbraculata	28 ± 1.8[b]	50	46 ± 1.6[a]	14 ± 1.6[c]	50	46 ± 0.7[a]

The semi-synthetic diet used by Popay (2001) is an effective food source for porina but the recipe includes clover as an essential ingredient, which was one of the target hosts for the planned host plant preference and performance experiments. Removing the clover from this semi-synthetic diet led to high larval mortality (data not shown) so an alternative was required. Carrot was cheap, readily

available and removed any risk of preconditioning the porina larvae with a plant host to be used for subsequent experiments.

Ferguson & Crook (2004) reported that egg hatch time decreased with an increase in temperature from 10–20℃ for *W*. *cervinata* and *W. copularis*. The results of the current study supported these earlier findings since eggs of all species hatched more quickly at the higher of the two temperatures examined (22℃ compared with 15℃, Table 4.1). Also, *W. cervinata* eggs hatched faster than the other two species at both temperatures (15℃: $F_{2,12}$ = 4.39, P=0.018; 22℃: $F_{2,12}$ = 26.92, P<0.001) whereas *W. copularis* was slowest. Ferguson and Crook (2004) also found that *W. cervinata* eggs hatched earlier than *W*. *copularis* eggs when reared at 10, 13, 16 and 20℃, with *W. cervinata* and *W. copularis* eggs hatching in 15 and 18 days respectively at 20℃. Importantly, the current study presents the first data on development of *W. umbraculata*. Larval survival was high (15℃: 47, 45, 46 and 22℃: 45, 46, 46 for *W. cervinata*, *W. copularis* and *W. umbraculata* respectively, with no significant difference in larval survival (15℃: $F_{2,12}$ = 2.50, P=0.124; 22℃: F2,12 = 0.769, P=0.485) at either temperature (Table 4.1).

Ferguson & Crook (2004) reared larvae individually, which is considerably more time consuming than the mass-rearing approach of 50 larvae per container used here. Also, they reported larval survival rates at pupation of 61-68% whereas the survival rate at 8 weeks in the current study was 90%. The rearing method presented here is also cheaper (as it did not require the purchase of specialised chemicals) and simpler (as it did not involve multiple ingredients and takes much less time to prepare) than previous methods. Also, the higher temperature (22℃) used here resulted in faster hatching of eggs compared with earlier studies (10–20℃). This method has the added advantages that carrot is always available and it does not pre-condition larvae to a common pasture species (clover), which is important for experiments focused on host plant interactions in porina.

4.5 Acknowledgments

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Chapter 5

The development, preference and host shift of porina on exotic and native plant hosts

5.1 Abstract

The success of the endemic pasture pests, *Wiseana* spp., provides a rare opportunity to understand the mechanism underpinning the evolution of their diet breadth and ask the question which *Wiseana* species have adapted to exotic pastures. This study investigated the fitness response of three species of *Wiseana* on putative native and exotic host plants using non-choice, host-shift and the preference assays. The results suggest that *W. copularis* and *W. cervinata* have undergone a host-range expansion (ability to use equally both a native and new host) onto exotic hosts rather than a host shift (loss of fitness on the ancestral host in comparison to the new host), while *W*. *umbraculata* has not. The mechanism that underpins this process is most probably 'ecological fitting' followed by a genetic trade-off.

Key words: *Wiseana copularis*, *W. cervinata*, *W*. *umbraculata*, native host, host-shift, host expansion

5.2 Introduction

The widespread replacement of native ecosystems by productive land sometimes results in outbreaks of native species (Lefort *et al*. 2015). Also, the movement of crops into new countries and regions due to commercial agricultural activity presents an opportunity for native insect herbivores to encounter new plant species, which results in unplanned natural host selection experiments (Murdoch *et al*. 2014). Plant introductions to novel habitats have occurred worldwide over hundreds of years to sustain human migrations and subsequent needs (Burnett *et al*. 2012). However, with ever-increasing globalisation of people and products, such introductions have shifted from species that mainly sustain food production (Godfray *et al*. 2010) to those introduced accidentally (McNeill *et al*. 2011) or planted for recreation (Brasier 2008). As a result, a large variety of complex relationships with members of native communities have thrived (reviewed by Cox 2004) with these exotic plants accumulating more new herbivorous species as time elapses after arrival (Brandle *et al*. 2008). These encounters may lead to expansions in dietary range (Singer *et al*. 1993, Carroll *et al*. 1998, Mack *et al.* 2000) as native insects succeed in colonizing exotic host plants. Alternatively, there could be a host-shift where native insects form an association with novel host plants (Janzen 1985, Holder 1990, Agosta 2006, Diegisser *et al*. 2009). The association of a phytophagous species with a novel host plant, where that species is able to use the new as well as the ancestral host is known as a host-range expansion (Mack *et al*. 2000, Janz

et al. 2001). Host-range expansions do not result in any fitness compromises. This allows the insect herbivore to use both its new and ancestral hosts (Diegisser *et al*. 2009), without generating unfavourable fitness response effects (Lefort *et al*. 2014) in the population. Conversely, a host-shift, host-switching or host-transference describes any situation whereby a population of insect herbivores forms an association with a novel host plant (Holder 1990, Agosta 2006), and are unable to use its new and ancestral host simultaneously (Diegisser *et al*. 2009). This can be detected by host-plant associated fitness trade-offs on the ancestral host (Via 1990, Diegisser *et al.* 2009). Only recently has it been formally acknowledged that native species can occasionally reach the status of `pest' or `invasive species' within their own native range (Lefort *et al*. 2014).

In New Zealand exotic pastoral plants were introduced in the 1800s. These introductions have resulted in diet alteration by the endemic lepidopteran genus, *Wiseana* (Hepialidae) commonly known as porina, such that it has reached pest status (Pottinger 1975, Dugdale 1994, Richards *et al.* 1997, Barratt *et al*. 1990, Zydenbos *et al*. 2013, Lefort *et al*. 2015). The larvae of this endemic insect feed on the foliage and shoots of the exotic hosts, ryegrass (*Lolium* spp.) and white clover (*Trifolium repens*). As a consequence, the species is ranked as a major economic pest of New Zealand pastures (Pottinger 1975, Richards *et al.* 1997). By widely replacing native ecosystems with more economically productive pastures, the natural balance in the herbivore-plant system has resulted in porina succeeding in colonizing exotic pastures outside their native host range. This anthropogenically derived modification has created novel ecological conditions that are apparently beneficial to porina (Lefort *et al*. 2015), but possibly detrimental to other native species.

Although porina have been reported to feed on 27 plant species (See various authors in Spiller & Wise, 1982, White 2002), only *Chionochloa rubra* (Kelsy 1968), *Phormium tenax* (Kirk & Cockayne 1909, Atkinson 1921, Miller 1971), *Carex secta* (Dugdale 1994) *Dicksonia squarrosa* (Hudson 1928) and bryophytes (White 2002) are natives. It has not been determined what caused *Wiseana* to expand its range into exotic pastures because its native hosts are not known (Sarah Mansfield, personal communication, AgResearch NZ), which makes “host” related studies difficult. No detailed studies have been done of the life history of porina on any of the putative native or exotic host plants and the full extent of their host range has not been investigated. There are currently five hypotheses in the literature that could explain why porina have become invasive on exotic pastures: (1) genetic trade-offs in performance on different hosts (antagonistic pleiotropy, a negative correlation in fitness over environments) (Ehrlich & Raven 1964, Gould 1979, Futuyma & Moreno 1988, Jaenike 1990, Scheirs *et al*. 2005), (2) mutation accumulation (Levins 1968, Kawecki 1994), (3) a coevolutionary arms race between host plants and herbivores (Bergelson, *et al*. 2001, Grosman *et al*. 2015), (4) ecological fitting (Janzen 1985, Agosta 2006); and (5) the superior-host hypothesis (Jaenike 1978, Courtney & Kibota 1990, Valladares & Lawton 1991, Scheir *et al*. 2000, Mayhew 2001, Clark *et al*. 2011).

According to the genetic trade-off hypothesis, different host plants impose different selection regimes on herbivores and this leads to different adaptations (Futuyma & Moreno 1988, Jaenike 1990, Fry 1996, Thompson 1996), such that the ability to adapt to one host results in a poorer performance on the alternative hosts (Gould 1979, Jaenike 1990, Rausher 1984) due to antagonistic pleiotropy among genes (Scheirs *et al*. 2005). These genetic trade-offs may explain the predominance of specialists among herbivorous insects (Jaenike 1990, Fry 1996, Scheirs *et al*. 2005) and may play an important role in sympatric speciation due to disruptive selection (Dieckmann & Doebeli 1999, Kondrashov & Kondrashov 1999). Sympatric speciation is a possible evolutionary route for species of *Wiseana* which overlap geographically.

The mutation accumulation hypothesis assumes that the adaptation to a host and loss of adaptation to an alternative host are not causally related (Kawecki 1994). Instead, this hypothesizes that herbivores will become less adapted to alternative hosts when specializing on one host because of the stochastic accumulation of mutations that are neutral on the current host, but result in reduced performance on the alternative hosts (Levins 1968, Kawecki 1994). The adaptation to the current host occurs simultaneously with the accumulation of mutations, but not necessarily at the same rate and may result in a negative correlation between performance on the native host and the novel host, although reduced performance on the native host is not the direct result of adaptation to the novel host. Hence, such a negative correlation could be erroneously interpreted as evidence of a genetic trade-off (Grosman *et al*. 2015).

The coevolutionary arms race between host plants and herbivores hypothesizes that host plants themselves may also evolve in response to herbivory, resulting in reduced adaptation of the herbivores (Bergelson *et al*. 2001). Herbivores on a novel host plant engage in an arms race with the novel host and continue to adapt to this new host, but not to its native host(s), because of a simultaneous coevolutionary arms race with other populations of herbivores (Grosman *et al*. 2015). This implies that a lack of competition from other herbivores on the novel host relative to the original host, encourages adaptation to the novel host. This process will also result in reduced adaptation of the herbivore to the native host, irrespective of the accumulation of mutations in the herbivore and without a genetic trade-off being involved (Grosman *et al*. 2015).

The ecological fitting concept proposes that herbivores colonize and persist in novel environments, use novel plants, and/or form novel associations with new host plant as a result of the suites of traits that they carry at the time of their encounter (Janzen 1985, Agosta 2006). Ecological fitting is an important process in structuring plant-insect associations and it represents an additional mechanism to cospeciation, coevolution and on-site evolution and persistence of species associations. However,

it differs from those processes in that evolution by either member of an association is not a prerequisite for its formation or persistence (Agosta 2006).

The superior host theory (also known as the optimal oviposition theory (Jaenike 1978), naïve adaptionist theory (Courtney & Kibota 1990), 'mother knows best' principle (Valladares & Lawton 1991) and the preference-performance hypothesis (Clark *et al*. 2011) all suggest that herbivore's adaptation to a novel host may be due to the host's superior quality, such as higher nutrient availability (Kawecki & Ebert 2004), relative to the original host. Adaptation can only result when performance of the herbivore on the novel host plant has improved the herbivore over generations, such that it performs better on that host than conspecifics that have remained on the native host (Grosman *et al*. 2015). This hypothesis relates to insects whose immature life stages are sedentary and have little or no ability to relocate. They are thus reliant on the host-plant choice of the mother. To maximise insect fitness, the hypothesis predicts a positive correlation between oviposition preference and offspring performance (Clark *et al*. 2011).

Presented here is the first comparative study on larval survival, adult emergence and life span of three porina species, *Wiseana cervinata*, *W*. *copularis* and *W*. *umbraculata,* on putative native and exotic host plants. This study also looks at the preference of *W*. *copularis* larvae for the putative native or exotic hosts and a host-shift experiment of *W*. *copularis* from a putative native host to the exotic hosts. The purpose of these studies was to identify native hosts of porina, describe the development of these porina species on these host plants, and to use the comparison between confirmed native hosts and exotic host plants to identify the probable mechanisms for the success of porina on New Zealand pastures: genetic trade-offs in performance, mutation accumulation, coevolutionary changes in the native host plant, ecological fitting, or superior host quality of the exotic pasture.

5.3 Material and methods

5.3.1 Plant species

Seven plant species across four families (Table 5.1) were selected for the no-choice, choice and host-shift tests. Five of the putative host plants selected were natives and their selection was based on literature, knowledge of plant diversity in tussock grasslands, size of the experiment and ease of sourcing for these plants. The other two species were the introduced pasture plants, white clover (*Trifolium repens*) and ryegrass (*Lolium perenne* × *Lolium multiflorum*). These exotic hosts were chosen because they are commonly sown and attacked by porina in New Zealand pastures. Young native plants were sourced from Plantlife Propagators Ltd (Ashhurst, New Zealand), while seeds of *L*. *perenne* × *L*. *multiflorum*, a hybrid (nil endophyte - because endophyte ryegrass is a more recent development

in New Zealand pastures) was obtained from AgResearch Ltd (Hamilton, New Zealand) and seeds of *T. repens* were obtained from the Lincoln University Field Services Centre (Lincoln, New Zealand).

Table 5.1: Putative native and exotic host plant species used in the study.

Plants	Common names	Family	Group
Festuca actae	Banks Peninsula blue tussock	Poaceae	Native
Chionochloa rubra	Red tussock	Poaceae	Native
Poa cita	Silver tussock	Poaceae	Native
Aciphylla squarrosa	Spear/sword grass	Apiaceae	Native
Phormium tenax	Flax	Asphodelaceae	Native
Trifolium repens	White clover	Fabaceae	Exotic
Lolium perenne × *Lolium multiflorum*	Ryegrass	Poaceae	Exotic

Young native plants were carefully transferred into 1.5 L pots containing four-month-old potting mix comprising of 80% bark, 20%, Osmocote Exact fertiliser (16 N:3.5 P:10 K), horticultural lime, hydroflo and pumice. Seeds of *L. perenne* × *L. multiflorum* and *T. repens* were grown in a greenhouse (Lincoln University, New Zealand) in 500 ml pots, ¾ filled with potting mix as described above and allowed to grow for 2 months prior to the porina feeding experiment (Figure 5.1).

Figure 5.1: Plants used for the experiments

These plants were also used for nutrient content analysis and metabolite profiling in Chapter 6 and larval foraging behaviour of porina in Chapter 7.

5.3.2 No-choice experiment of *Wiseana* species on exotic and native host plants

The experiment was conducted in a constant temperature (CT) cabinet (Contherm, Model Cat.190.RHS) at the Field Ecology Laboratory, Lincoln University. The growth chamber was maintained at 15℃ (s.d. ± 1.49℃) with a photoperiod of 12L: 12D, light intensity of 500 $\mu mol^{-2}\ s^{-1}$ (s.d. ± 10 $\mu mol^{-2}\ s^{-1}$) during the 12-hour photoperiod and a relative humidity at 90% (s.d ± 3%).

Following molecular species identification of the female parent (Chapter 3), eight weeks old larvae of *W. copularis* (*n* = 210), *W. cervinata* (*n* = 63) and *W. umbraculata* (*n* = 63), that had been reared using the methods of Atijegbe *et al*. 2017 (Chapter 4) were weighed and placed in individual 120 ml plastic containers ½-¾ filled with Grade-2 moist horticultural bark (HB). Larvae were ordered from the lowest to the highest weight to allow for the effect of weight-related confounding factors. Twenty-one containers were randomly allocated to a tray to create a block (Figure 5.2a), with a container randomly allocated to one of seven plant host treatments: *A. squarrosa* (T1), *C. rubra* (T2), *F. actae* (T3), *P. tenax* (T4), *P. cita* (T5), *L. perenne* x *L. multiflorum* (T5) and *T. repens* (T7). Each treatment was replicated three times within a block. Larvae from each block were siblings hatched from eggs from the same mother. There were 16 blocks in total, which consisted of 10 blocks of *W. copularis*, 3 blocks of *W. cervinata* and 3 blocks of *W. umbraculata* (Figure 5.2b). Feeding trials were performed at 15℃ over a period of 57 weeks until larvae pupated. Larvae were fed *ad libitum* with freshly cut foliage/shoots of the selected host plants twice weekly. The larvae were checked visually each day for mortality and the moist HB changed once a month when larvae were weighed.

Figure 5.2: a) A block set-up with 21 containers. b) Blocks set-up in the CT cabinet

The fitness response within and between females was evaluated by measuring survivorship of larvae, percentage increase in weight on a monthly basis, number pupated, pupal weight, and adult emergence.

5.3.3 Feeding preference of *W. copularis* - native vs exotic host choice test

This was conducted in a growth chamber measuring 2.36 m × 1.57 m × 2.11 m (Conviron, Model BDW40) equipped with Elite Agro ceramic metal halide lamp (Model 930, 355W, Philips) canopy with a clear glass barrier, and a downward airflow distribution system using an additive control to provide ambient CO_2 conditions inside the room. The room temperature was maintained at 15℃ (s.d. ± 0.9℃) with a photoperiod of 12L: 12D. Photosynthetically active radiation at 1 m below the lamp canopy was 500 $\mu mol^{-2}\ s^{-1}$ (s.d. ± 10 $\mu mol^{-2}\ s^{-1}$) during the 12-hour photoperiod with relative humidity maintained at 90% (s.d ± 3%). Two metal benches (2 m × 0.5 m × 0.9 m) were placed on the floor on which the experiment was set up (the arena).

Eight weeks old *W. copularis* larvae (*n* =121), reared using the methods of Atijegbe *et al*. 2017 (Chapter 4), were arranged from largest to smallest and each larva randomly allocated to a plastic container (17 cm x 17 cm x 8.8 cm), ½ filled with moist HB. The larva was placed in an artificial burrow made at the centre of the container by drilling a 5 cm deep hole into the media with a 0.5 cm diameter stick. At equal distance from the centre, five pieces of cut foliage or shoot (~2 cm long) from one of the native plants: *A. squarrosa, C. rubra*, *F. actae*, *P. tenax* or *P. cita,* were placed in a close-set pile at one end and five pieces of an exotic host: *L. perenne* × *L. multiflorum* or *T. repens,* was placed at the other end (Figure 5.3). A paired choice of the two exotics, *L. perenne* × *L. multiflorum* and *T. repens* was also done. For each pair, the the bioassay was replicated 11 times. At the beginning, the weight of all pieces in a pile were weighed and the average weight of a piece of foliage or shoot determined and then the feed trials performed for 14 days at 15℃. The number of pieces of foliage consumed by the larva was recorded every 42 hours, after which the plant material was replaced with five fresh pairs of foliage pieces.

Figure 5.3: a) Set-up of choice test. b) Set-up of choice test after 24 hours.

5.3.4 Host-shift bioassay of *W. copularis* larvae from native to exotic host plants

This was also conducted in a CT cabinet as described in section 5.3.2. Eight-week old *W. copularis* larvae (*n* =150) from five mothers, reared using the methods of Atijegbe *et al*. 2017 (Chapter 4), were weighed and placed in individual 120 ml plastic containers ½-¾ filled with moist HB. Containers were randomly allocated to five trays so as to create five blocks, with larvae ordered from the lowest to the highest weight on the trays to allow for confounding weight-related factors. Each container was randomly assigned to a feeding treatment. Feeding trials were performed at 15°C, with a 12L: 12D photoperiod over a period of 24 weeks. This corresponds to the most intense feeding period of the porina larval stage. All larvae were fed *ad libitum* with freshly cut foliage/shoots of the assigned native host plant. They were either fed with *P. cita*, *C. rubra*, *F. actae*, *P. tenax* or *A. squarrosa* for 12 weeks respectively for treatments 1 (T1), 2 (T2), 3 (T3), 4 (T4) and 5 (T5), followed by a shift after 12 weeks to the exotic host plants. Ten containers from each treatment were shifted to *T. repens* and *L. perenne* × *L. multiflorum*, while 10 remained on the initial treatment (serving as controls) for another 12 weeks. The fitness response of the larvae was evaluated by measuring survivorship and percentage increase in weight on a weekly basis.

5.3.5 Statistical analyses

Survival analyses were performed using the Kaplan-Meier estimator, while larval weight was analysed using analysis of variance (ANOVA). Larval preference was analysed using paired sample *t*-tests, while data from the host-shift were analysed using a repeated measures ANOVA. Statistical tests were conducted using SigmaPlot 14 (SYSTAT Software Inc, USA) and GenStat 18 (GenStat®, VSN International Ltd, UK) statistical packages.

5.4 Results

5.4.1 Fitness of *Wiseana* species on exotic and native host plants in a no-choice experiment

A total of 294 larvae, *W. copularis* (*n* = 210), *W. cervinata* (*n* = 42) and *W. umbraculata* (*n* = 63) were used in the experiment. One block of *W. cervinata* (*n* = 21) was excluded because the larvae all died of disease passed down from their mother. Survival of *W. copularis* larvae was significantly different amongst the different plant species (Kaplan-Meier, *P* <0.001). Those fed on the exotic, *T. repens* exhibited higher survivorship than those on the other plants except for larvae fed *F. actae* (Figure 5.4a, Table 5.2). The larval development of *W. copularis* was longer on *F. actae* and took 283 days (Table 5.3), while the pupal development was longest on *L. perenne* × *L. multiflorum* with 38 days (Table 5.3). Larvae from *T. repens* were heavier and statistically different from the larvae

of other host plants (Table 5.4). Pupae from *T. repens* were heavier for those larvae that pupated (Table 5.4). Emergence of the adults occurred only in larvae fed *A. squarrosa* (3%), *F. actae* (3%), *L. perenne* × *L. multiflorum* (7%) or *T. repens* (40%), with adults from *L. perenne* × *L. multiflorum* being heavier than other emerged adults (Table 5.3).

Equally, larval survival of *W. cervinata* was significantly different between the plant species (Kaplan-Meier, P <0.001). Those feed on the exotics *T. repens* exhibited higher survivorship compared to the native *F. actae* (Figure 5.4b, Table 5.2). Although, larvae from *T. repens* were heavier, they were not statistically different from the larvae of *F. actae*. However, larvae of *T. repens* and *F. actae* were significantly different in weight from larvae of the other host plants (Table 5.4). Larval and pupal development was longer on *T. repens* (Table 5.3). However, adults emerged only from these two plants, *T. repens* (83%) and *F. actae* (20%). The pupae and adults from *F. actae* were heavier compared to *T. repens* (Table 5.4).

As for the other two species, survivorship of *W. umbraculata* larvae was significantly longer on *T. repens* (Kaplan-Meier, P = 0.01) (Figure 5.3c, Table 5.3). Larvae from *T. repens* were heavier than larvae from other hosts before they all died (Table 5.4).

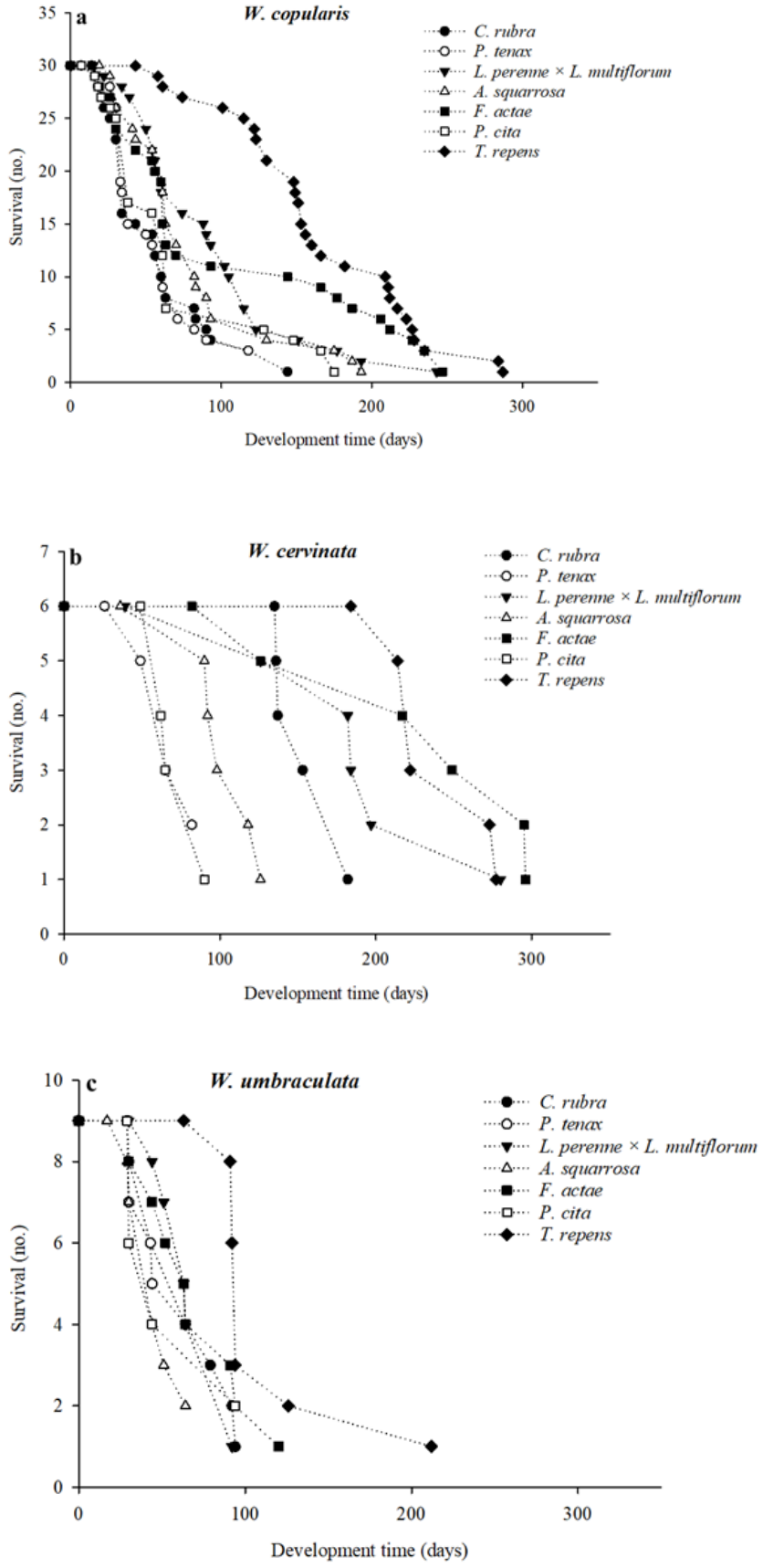


Figure 5.4: Survival curves for larvae on the different plant species for a) *W. copularis*, b) *W. cervinata* and c) *W. umbraculata*.

Table 5.2: Pairwise comparison of survival statistics for *Wiseana* spp. larvae on native and exotic plants

W. copularis	***F. actae***	***C. rubra***	***P. cita***	***A. squarrosa***	***P. tenax***	***T. repens***
F. actae	-					
C. rubra	8.22 ns	-				
P. cita	5.78 ns	1.21 ns	-			
A. squarrosa †	2.18 ns	3.88 ns	2.44 ns	-		
P. tenax	8.32 ns	0.03 ns	1.28 ns	5.00 ns	-	
T. repens †	4.30 ns	49.02 ***	28.59 ***	26.88 ***	51.7 ***	-
L. perenne × *L. multiflorum* †	0.69 ns	8.26 ns	3.60 ns	1.30 ns	9.42 *	17.21 ***
W. cervinata						
F. actae †	-					
C. rubra	2.79 ns	-				
P. cita	9.77 *	11.92 *	-			
A. squarrosa	5.83 ns	12.09 *	5.30 ns	-		
P. tenax	9.32 *	11.39 *	0.10 ns	6.33 ns	-	
T. repens †	0.49 ns	11.78 *	11.92 **	12.09 *	11.39 *	-
L. perenne × *L. multiflorum*	1.80 ns	2.21 ns	6.26 ns	5.83 ns	6.33 ns	0.74 ns
W. umbraculata						
F. actae	-					
C. rubra	0.46 ns	-				
P. cita	1.11 ns	0.02 ns	-			
A. squarrosa	3.27 ns	2.13 ns	0.23 ns	-		
P. tenax	1.31 ns	0.88 ns	<0.01 ns	0.73 ns	-	
T. repens	3.83 ns	5.64 ns	4.69 ns	15.77**	13.04**	-
L. perenne × *L. multiflorum*	0.48 ns	0.04 ns	0.28 ns	2.92 ns	0.63 ns	9.20 *

ns = Non significant, * = Significant at $p < 0.05$, ** = Significant at $p < 0.01$, *** = Significant at $p < 0.001$, † Adult(s) of *Wiseana* spp. emerged from larvae fed on these plants

Table 5.3: Mean development time of immature stages of *Wiseana* on the host plants.

Species	Host plant	Larval stage (days)	Pupal stage (days)
W. copularis	*F. actae*	283	23
	C. rubra	-	-
	P. cita	-	-
	A. squarrosa	249	33
	P. tenax	-	-
	T. repens	254	33
	L. perenne × *L. multiflorum*	241	38
W. cervinata	*F. actae*	290	30
	C. rubra	-	-
	P. cita	-	-
	A. squarrosa	-	-
	P. tenax	-	-
	T. repens	349	34
	L. perenne × *L. multiflorum*	-	-
W. umbraculata	*F. actae*	-	-
	C. rubra	-	-
	P. cita	-	-
	A. squarrosa	-	-
	P. tenax	-	-
	T. repens	-	-
	L. perenne × *L. multiflorum*	-	-

Table 5.4: Pupation number, pupal weight, adult emergence and adult weight for *Wiseana* spp. on native and exotic plants

W. copularis	***n***	**Larval weight (mg)**	**No. of pupae**	**Pupal weight (mg)**	**Adult emergence**	**Adult weight (mg)**
F. actae	30	6.68[b]	5	0.25[c]	1	0.19[b]
C. rubra	30	1.77[b]	0	0	0	0
P. cita	30	2.24[b]	0	0	0	0
A. squarrosa	30	2.59[b]	1	0.34[b]	1	0.18[b]
P. tenax	30	1.65[b]	0	0	0	0
T. repens	30	17.10[a]	13	0.46[a]	12	0.22[b]
L. perenne × *L. multiflorum*	30	4.31[b]	3	0.34[b]	2	0.28[a]
W. cervinata						
F. actae	6	36.30 [a]	3	0.58[a]	1	0.32
C. rubra	6	13.40[b]	0	0	0	0
P. cita	6	2.95[b]	0	0	0	0
A. squarrosa	6	7.27[b]	0	0	0	0
P. tenax	6	2.31[b]	0	0	0	0
T. repens	6	45.60[a]	5	0.46[b]	5	0.19
L. perenne × *L. multiflorum*	6	18.30 [b]	0	0	0	0
W. umbraculata						
F. actae	9	4.16[b]	0	0	0	0
C. rubra	9	3.13[b]	0	0	0	0
P. cita	9	1.75[b]	0	0	0	0
A. squarrosa	9	1.57[b]	0	0	0	0
P. tenax	9	1.43[b]	0	0	0	0
T. repens	9	16.70[a]	0	0	0	0
L. perenne × *L. multiflorum*	9	2.23[b]	0	0	0	0

5.4.2 Feeding preference of *W. copularis* larvae

The average quantity of herbage consumed by the larvae for each of the plant species was as follows: *A. squarrosa* (76.0 mg), *C. rubra* (55.0 mg), *F. actae* (9.3 mg), *P. tenax* (34.0 mg), *P. cita* (12.2 mg), *L. perenne* × *L. multiflorum* (16.7 mg) and *T. repens* (34.8 mg).

A series of paired sample t-tests were conducted to compare the preference of *W. copularis* larvae, measured as weight of plant material eaten, when offered a choice of various combinations of exotic and native plant species, or between the exotic plant species (Table 5.4) The results showed that when offered a choice between the exotic plant *T. repens* and any of the native plant species, the larvae showed preference for *T. repens* only when *T. repens* was paired with *P. tenax*, *F. actae*, and *A. squarrosa* (Table 5.4). However, when offered a choice between the other exotic species *L. perenne* × *L. multiflorum* and any of the native plant species, or between the two exotics *T. repens* and *L. perenne* × *L. multiflorum*, the larvae showed no detectable preferences (Table 5.4). A similar preference analysis based on the number of plant pieces taken gave a similar result (Appendix A).

Table 5.5: Quantity eaten by *W*. *copularis* larvae offered choices of exotic and native plants and analysed using a paired t-test

Exotic plants	**Mean ± se (mg)**	**Native plants**	**Mean ± se (mg)**	***n***	**t**	**df**	**P**
T. repens	108.00 ± 20.00	*P. tenax*	26.30 ± 15.80	11	3.20	10	0.005
T. repens	66.40 ± 30.60	*A. squarrosa*	0.00 ± 0.00	11	2.18	10	0.042
T. repens	71.20 ± 23.90	*F. actae*	3.80 ± 2.57	11	2.80	10	0.011
T. repens	49.00 ± 20.00	*C. rubra*	36.20 ± 17.60	11	0.48	10	0.637
T. repens	31.60 ± 15.9	*P. cita*	8.89 ± 3.34	11	1.40	10	0.176
Exotic plants	**Mean ± se (mg)**	**Native plants**	**Mean ± se (mg)**	***n***	**t**	**df**	**P**
L. perenne × *L. multiflorum*	8.34 ± 4.05	*P. tenax*	6.95 ± 4.10	11	0.24	10	0.813
L. perenne × *L. multiflorum*	9.47 ± 5.03	*A. squarrosa*	53.50 ± 32.50	11	-1.34	10	0.195
L. perenne × *L. multiflorum*	6.06 ± 2.54	*F. actae*	5.50 ± 5.05	11	0.10	10	0.921
L. perenne × *L. multiflorum*	6.06 ± 2.54	*C. rubra*	90.00 ± 54.60	11	-1.54	10	0.141
L. perenne × *L. multiflorum*	3.03 ± 2.03	*P. cita*	1.11 ± 1.11	11	0.83	10	0.453
Exotic plant	**Mean ± se (mg)**	**Exotic plant**	**Mean ± se (mg)**	***n***	**t**	**df**	**P**
T. repens	30.10 ± 14.00	*L. perenne* × *L. multiflorum*	12.10 ± 4.41	11	-1.22	10	0.237

5.4.3 Host-shift bioassay of *W. copularis* larvae from native to exotic host plants

Larvae of *W*. *copularis* fed initially on a native plant for 12 weeks then artificially transferred to an exotic host plant showed different weight gain trends depending on the plant species involved (Figure 5.5).

In figure 5.5a, the three groups fed initially on *A*. *squarrosa*, there was little overall weight gain over the initial 12 week period and no significant differences amongst them. However, this changed substantially after the host-shift with the larvae shifted to *L*. *perenne* × *multiflorum*, rapidly increasing weight by over 200% for the remaining 12 weeks, while those on *T*. *repens* or those on *A*. *squarrosa* (control) lost weight and died after three weeks. Similarly, the three groups of larvae initially fed with *C. rubra* gained little weight in the first 12 weeks with no significant difference between them. Then over the next 12 weeks, the weight of those shifted to *T*. *repens* and the controls remaining on *C. rubra* declined until death three weeks later. However, as for the *A*. *squarrosa* group above, those shifted from *C. rubra* to *L. perenne × multiflorum* rapidly increased weight by over 175%.

Conversely, when *W. copularis* larvae were initially fed *F. actae*, the three groups all steadily gained weight to a similar level up to week 12 and continued to gain weight to over 100% post-shift to the same degree for exotic and control plants for the remaining 12 weeks (Figure 5.4c). However, while there was no difference in weight gained between the larvae shifted to *L. perenne* × *L. multiflorum* and those that remained on *F. actae*, the *T. repens* weight gain was significantly greater than the controls (P = 0.034). A similar but more subtle trend of increased weight gain was observed for larvae initially fed on *P. cita* (Figure 5.4d). Only slight increases were recorded for all three groups in the first 12 weeks and continued as such for the control in the second 12 weeks. In contrast to that, with the shift from *P. cita* to either exotic, *L. perenne* × *L. multiflorum* or *T. repens,* the larvae showed a considerable gain in weight to ~200% with no detectable difference in weight between these groups (Figure 5.4d). Here there was a significant difference in larval weight among the groups (P <0.001).

Finally, *W. copularis* larvae that were initially fed on *P*. *tenax* all died four weeks pre-shift (data not shown). Death was caused by starvation as a result of a latex-like liquid exuded by the plant as the larvae fed. This coagulated on exposure to air and stuck their mouthparts together, thus preventing feeding.

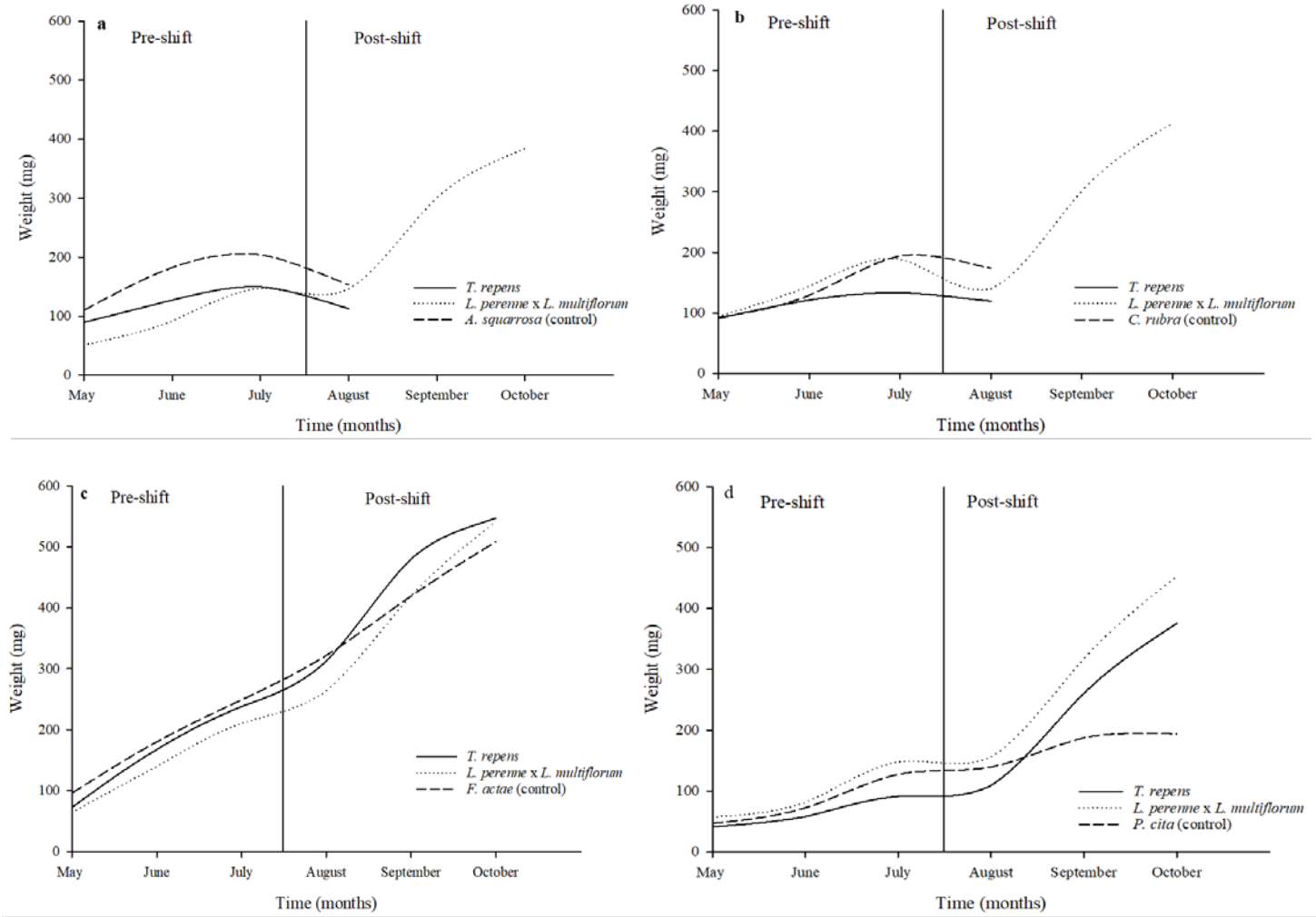


Figure 5.5: Cumulative weight gain of *W. copularis* larvae following 12 weeks feeding on a native plant then transferred for 12 weeks to an exotic plant, a) *A. squarrosa*, b) *C. rubra*, c) *F. actae* or d) *P. cita*.

5.5 Discussion

The widespread introduction of important agricultural crops such as cacao and sugarcane offer spectacular examples of how rapidly plants can recruit herbivores (Agosta 2006). Thus, understanding such insect/host plant relationships has been at the core of research for ecologists, evolutionary biologists and entomologist for decades. Of particular interest is understanding the mechanism that underpins the invasive success of insect herbivores outside their native range. It is challenging enough trying to discern the process when the ancestral host(s) are known, but becomes extremely difficult when the ancestral plant hosts are unknown, as is the case with porina. For a native plant to be considered an ancestral host of porina, the larvae should be able to complete their life cycle while feeding on that plant and be able to produce viable adults (Dhooria 2009, Gordh & Headrick 2011). This study aimed to address this gap in knowledge of the native hosts of porina prior to their now seemingly exclusive use of exotic plants. It also aimed to identify the drivers of success of *Wiseana* spp. in New Zealand pastures outside their native host range.

To address this, the present study aimed to assess the fitness, survivorship and preference for potentially original native hosts compared to their current exotic hosts. The outcome may then help to develop hypotheses as to which plants might have been an ancestral host to *Wisiana* spp. Using native plants chosen based on literature and knowledge of the diversity of plants in the tussock grasslands, the results showed the presence of strong intra-specific variations in fitness of three *Wiseana* species. This was expressed as a conspicuous diversity in survivorship, larval weight increase, pupal weight, adult weight and adult emergence when their larvae were reared on the putative native or exotic host plants. The completion of insect development and adult emergence is a strong indicator of the suitability of a plant species as a host (Dhooria 2009, Gordh & Headrick 2011). Here, for *W*. *copularis*, this occurred with both of the exotics, *T. repens* and *L. perenne* × *L. multiflorum*, and two native species, *F. actae* and *A*. *squarrosa*. Emergence of *W*. *cervinata* on the other hand occurred only on *T. repens* and *F. actae*. The growth response of field collected larvae of *Wiseana* spp. fed on native plants, *L. perenne* × *L. multiflorum* and a mixed grass diet (Atijegbe *et al*. 2017, Chapter 3), also suggest that *F. actae* and *A. squarrosa* are native host plants of *Wiseana*. Although rearing to demonstrate reproductive capability and therefore full completion of the lifecycle was not possible here because of time constraints, this does allow the hypothesis to be presented that *F. actae* might have been a native host of *Wiseana*, as both *W*. *copularis* and *W*. *cervinata* successfully reached adulthood on this species. On the basis of *W*. *copularis* development, *A. squarrosa* may have been an ancestral host. Consistent with successful development, general fitness of *W*. *copularis* and *W*. *cervinata* was observed to be high on *T*.

repens. This was expressed as high survivorship, high larval weight increase, number of pupae, pupal weight, adult emergence and adult weight. Interestingly, *W*. *umbraculata* also showed high fitness performance on *T*. *repens* before they died.

These results may reflect to some degree a combination of hypotheses that explain why porina has become successful on exotic hosts within its home range. In the case of *W*. *cervinata* and *W*. *umbraculata* the theory of mutation accumulation (Levins 1968, Kawaecki 1994) may apply. There, herbivores become less adapted to the native hosts as they specialize on the novel host, accumulating random mutations that are neutral on the novel host but result in a reduced performance on the native host. For *W. umbraculata* the coevolutionary arms race principle between the native and exotic hosts (Bergelson *et al*. 2001, Grosman *et al*. 2015) may apply, where the herbivores are continually adapting to novel host(s) and become less well associated with the native host because of a coevolutionary arms race with other populations of herbivores on the exotic hosts. This principle also posits a reduced adaptation of the herbivore to the native host without mutation accumulation in the herbivore or a trade-off. The superior host hypothesis might also explain the better performance of some *Wiseana* spp. on exotic plant species (Clark *et al*. 2011, Kawecki & Ebert 2004). Here the herbivores adapt to a novel host due to its higher nutrient availability allowing the herbivores to perform better on that host than conspecifics that remained on the native host (Grosman *et al*. 2015). Natural selection further shapes the relationship with the novel host over generational time. However, none of the mutation accumulation, the coevolutionary arms race or superior host hypotheses on their own can completely explain the increased performance of *Wiseana* spp. on the exotic hosts because the reduced performance on the native host was not expressed by the three species of *Wiseana* studied. This means the premise that *Wiseana* lacks competition from other herbivores is not valid (Grosman *et al*. 2015), and female *Wiseana* moths do not shown any specificity with respect to egg laying. Alternatively, it is possible that inherent mechanisms relying on high degrees of phenotypic plasticity, for instance derived from antagonistic pleiotropy among genes (Scheirs *et al*. 2005, Lefort *et al*. 2015), favour the genetic trade-off hypothesis (Futuyma & Moreno 1988, Jaenike 1990, Fry 1996, Thompson 1996) or ecological fitting principle (Agosta 2006) as partly or completely responsible for the high fitness performance observed in *Wiseana* spp., particularly on *T*. *repens*.

The term genetic trade-off, as defined by Fry (1996), is when adaptation to a host results in a relatively poor performance on alternative hosts due to antagonistic pleiotropic action of one or more genes. As a result, no genotype has maximal fitness on different hosts and eventually natural selection will promote host specialisation (Jaenike 1990, Fry 1996, Agrawal 2000). *Wiseana* spp.

inhabit the same geographic region so it is possible that they evolved from a surviving ancestral species, possibly by sympatric speciation (Dieckmann & Doebeli 1999, Kondrashov & Kondrashov 1999) as a result of disruptive selection. Phylogenetic analyses of the *Wiseana* genus by Brown *et al*. (1999) and (2000) using a combination of morphology, molecular COI & II ITS2 and allozyme data, showed a recent divergence of *W. copularis*, *W. cervinata* and *W. umbraculata* from a common *Wiseana* ancestor which gives further credence to the genetic trade-off hypothesis with *W. copularis* the more recent to diverge having a broader diet breadth. Worthy to note is that *W. umbraculata* is not considered a pest of pasture because the larvae of this species have not been recovered from infested pasture (S. Mansfield personal communication, AgResearch, NZ) which may explain why they did not develop on the exotic hosts.

Ecological fitting proposed by Janzen (1985) states that an insect herbivore colonise and persist on a novel host plant as a result of the suite of traits they carry at the time the encounter the novel host plant. Ecological fitting offers an alternative to the coevolutionary argument often used to explain species associations and the existence of widespread species that occupy very different environments (Janzen 1980), as the case with *Wiseana*. Moreover, Holder (1990) suggested that this type of association often arises because of the physical proximity of the ancestral and the new host-plant species. This is consistent with the current scenario following European settlement in New Zealand, when numerous native forests and grasslands were replaced by exotic pastures and crops (McDowall 1994, Lee *et al*. 2006). Effectively, this pattern of early settlement modification of the New Zealand landscape resulted in new ecological configurations where native grasslands ended up neighbouring exotic cultures and grass pastures. It is believed that this physical proximity has resulted in the contraction of native plant distribution ranges and in the consequent exploitation of the new neighbouring modified habitats by native herbivore species (Yeates 1991), as would explain the behaviour observed in *Wiseana* spp.

However, a more probable explanation for the exploitation of both native and exotic host plants by *W. copularis* and *W. cervinata* could be that these species have not yet undergone a host-shift but only a host-range expansion onto exotic pastoral plants. This explanation is likely because of the close relationship that exists between this process and that of ecological fitting, where no significant adaptation to the newly encountered exotic host is required (Diegisser *et al*. 2009, Agosta *et al*. 2010). Nevertheless, the differences in fitness performances between *W. copularis*, *W. cervinata* and *W. umbraculata*, as observed following their development on the hosts, seem to refute this possibility and suggest another explanation. The larvae of *W. umbraculata* seemed unable to benefit from these native hosts, as shown by low weight increase of the larvae, total

mortality of larvae and therefore non-emergence of viable adults. This indicates that *F. actae* and *A. squarrosa* are probably not ancestral hosts of *W. umbraculata*. Advancing this concept further implies that *W. copularis* and *W. cervinata* have undergone either a host-shift or host expansion from their ancestral host to the natives, *F. actae* and *A. squarrosa*, while *W*. *umbraculata* has not. Heard & Kitts (2012) suggested that host expansion can be followed by host-associated differentiation that can result in the evolution of new biotypes of specialist races, or so-called host-races (Diehl & Bush 1984, Drès & Mallet 2002).

This fitness compromise expressed as a host-plant associated fitness trade-off (Via 1990, Diegisser *et al*. 2009) resulting in some degree of maladaptation to the native host plant is compatible with the theory fitting hypothesis (Agosta 2006). Although the ecological fitting hypothesis seems to comply with this case study, the variation in larval performance of *W. copularis*, following the artificial host-shift from the native to exotic plants suggests that some level of evolutionary change has occurred in *Wiseana* spp. leading to a host-associated differentiation. Eventually this has resulted in the evolution of new biotypes of specialist races, or so-called host-races (Diehl & Bush 1984, Drès & Mallet 2002). Various examples of host-race formation in insects have been described. Amongst the most recent examples, Downey & Nice (2011) reported the probable formation of host-races in the juniper hairstreak butterfly (*Callophrys gryneus*), after observing differences in larval fitness performances when reared on natal versus alternate hosts. Bourguet *et al*. (2014) suggested that the emergence of the Asian and European corn borers from the genus *Ostrinia* (Lepidoptera, Crambidae) as two distinct species was as a result of ecological speciation from an ancestral species leading to reproductive isolation between them. Recently, Lefort *et al*. (2015) in a study of the feeding preference and fitness performance of the New Zealand scarab beetle *Costelytra zealandica* (White) and its congener *C. brunneum* (Broun), reported a strong intraspecific variation to suggest the existence of distinct host-races in the species. The preference of *T*. *repens* to the native hosts also confirms that a strong interspecific variation exist in the diet breadth of *W. copularis*.

The results of this study strongly suggest an ecological fitting followed by genetic trade-off could have led to the emergence of distinct species in the *Wiseana* genus. Phenotypic plasticity that initially facilitated host-race formation, could have been occurring over a long period, ultimately leading to speciation of *Wiseana* (e.g. Agrawal 2000, Agosta 2006, Matsubayashi *et al*. 2009, Heard & Kitts 2012). Furthermore, these findings point to a very interesting case of sympatric species formation facilitated by exotic plant introductions, and which has resulted in the rise of an endemic insect to the rank of invasive species outside its native range. The results from this study have

provided new insight into the mechanism(s) underpinning the invasion success of porina into exotic pastures throughout New Zealand. In summary, this study has shown evidence of (1) ecological fitting followed by a genetic trade-off resulting in a host range expansion in *Wiseana* spp., (2) the ability of *W. cervinata* to complete development on *F. actae* and *W. copularis* on *A. squarrosa* and *F. actae* which indicate that these might have been the native hosts of porina and (3) host-shift performance of *W. copularis* from native to exotic hosts plants shows that some level of evolutionary change has occurred in that species allowing it to exploit both its native and novel exotic hosts. In contrast, *W. umbraculata* has not yet evolved by any of these mechanisms to become invasive on exotics and presumably is only able to exploit its (unknown) ancestral host.

Chapter 6

Nutrient content and metabolite profiles of exotic and native host plants of porina.

6.1 Abstract

In the last hundred years, New Zealand native grasslands and forests have been modified extensively in producing pastures for the livestock industry and as a result the native herbivore, *Wiseana* have become pest on pasture. Seven host plants were investigated to test the hypothesis whether plant nutrient and structural compounds play a role in the success of *Wiseana* in exotic pastures. The results showed that the exotic grass, *Lolium perenne* × *Lolium multiflorum* contains the highest amount of silicon (16.66 g kg^{-1}), while the native monocotelydon, *P. tenax* contains the least (0.57 g kg^{-1}). Carbon was significantly higher in the native plants (≥ 43%) and lower in the exotic ones (≤ 41%), while the exotic hosts had higher nitrogen contents (≥ 4%) than the natives (≤ 2%). The acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents were lower in the exotic (ADF: ≤ 20%, NDF: ≤ 33%) and higher in the native plants (ADF ≥ 34%, NDF: ≥ 50%), respectively. As expected, in a hierarchical cluster analysis metabolites clustered together at the genus level, with a clear seperation between the exotic and native plants. This study shows that compared to potential native host plants, exotic pasture plants are rich in nitrogen and low in structural compounds. The results therefore suggest that these factors have been a key to the success of *Wiseana* in New Zealand pastures.

Key words: Exotic and native host, metabolites, carbon, nitrogen, carbon nitrogen ratio, silicon, acid detergent fibre, neutral detergent fibre.

6.2 Introduction

Before the arrival of humans to the shores of New Zealand, the vegetation was very diverse (Brougham 1978, Odgen 1998). The North Island was dominated by areas of podocarp/mixed hardwood forests, scrublands, fernlands and swamplands, with small areas of beech and beech/podocarp forest and a small area of tussock grassland on the central plateau; the South Island was predominately-lowland tussock grasslands with small areas of swamplands, fernlands and scrublands. The lowland tussock grasslands gave way with elevation to sub-alpine grasslands, to scrublands in the higher reaches of the Southern Alps, bounded by beech/podocarp forests to the west and a podocarp/mixed hardwood forests to the east (Brougham 1978). The last century

has seen a dramatic transformation of New Zealand with about 50% of the land area farmed at varying levels with 95% of the country's farmed lands devoted to pastoral farming, nearly two-thirds of which is in sown pastures (Anon. 2016), with an attendant increase in infestation of pastures by *Wiseana*. The improvement of pasture plants in New Zealand for about 100 years (Wratt & Smith 1983) have seen gains in the quality of forage, productivity, and resistance against insect pests (Easton *et al.* 1989, Easton *et al*. 1997, Woodfield 1999, Easton *et al*. 2001). However, an understanding of the interactions between *Wiseana* and its native host plants may suggest new breeding lines for pasture species against this herbivore.

Spiller & Wise (1982) quote various authors to have recorded porina feeding on a broad range of native plants. Later, Barratt & Patrick (1987) reported that *Wiseana* fed on native *Chionochloa* spp (*Chionochloa*: Poaceae). However, in all these studies, it was the non-feeding adults trapped within the study areas and the larval feeding life stage has not been directly linked to the supposed native host plants and none of the plants have been studied as hosts of porina (Chapter 5). As a result, White (2002) stated that the claim by Miller (1971) that porina feeds on the New Zealand native flax (*Phormium tenax*) was dubious. Presumably, the success of *Wiseana* in occupying the niches created by exotic pastures may be because of changes/modifications in their native habitats in New Zealand (Brown *et al*. 2000, Ferguson *et al*. 2017), and a particular ability to adapt such that the exotic pasture has become more suitable to porina than its native hosts (Murdoch *et al*. 2014, Lefort *et al*. 2015).

Phytophagous insect growth and survival are generally concentrated on host plants and/or plant species with either most suitable nutrient quality or quantity (Mattson 1980, Schowalter 1981), or greatest degree of availability (Cates 1980). Plants, like all other living things, also need food for their growth and development (Uchida 2000). Food(s) required for their nourishment and health are known as plant nutrients, which are essential in performing critical roles in plant growth and development. Plant analyses have been developed to primarily provide information on the nutrient status of plants (Smith & Loneragan 1997). The application of plant analysis as a diagnostic tool for nutrient content has undergone a lot of refinement (Goodall & Gregory 1947, Ulrich *et al*. 1959, Ulrich & Hill 1967, Bates 1971, Bouma 1983, Martin-Prevel *at al*. 1987, Munson & Nelson 1990, Jones 1991, Bergmann 1992). Advances in the capabilities of modern analytical systems such as the atomic absorption spectroscopy, inductively coupled plasma optical emission spectrometry (ICP-OES), X-ray fluorescence (XRF) and near infrared spectrometry have not only simplified procedures, but also broadened the scope of nutrients that can be measured (Smith & Loneragan 1997).

One means to characterise the status of a plants' nutrients, and thus its potential to meet the nutritional requirements of insect herbivores, is to consider its metabolome. The metabolome refers to the complete set of small-molecule chemicals found within a biological sample (Oliver *et al*. 1998, Desbrosses *et al*. 2005). The metabolome thus comprises all of the metabolites in a biological cell, tissue or organism. These metabolites are divided into primary metabolites that are directly involved in primary growth development and reproduction whereas secondary metabolites are indirectly involved in metabolisms while playing important ecological functions (Prins *et al*. 2009). No single analytical platform has yet been devised to measure all the metabolites in an organism and the quest for a single platform to unlock this Holy Grail of the plant metabolome is yet to be discovered. Instead various extraction, separation and detection systems have been optimised for the analysis of certain classes of compounds (Desbrosses *et al*. 2005). These include separation systems like gas chromatography (GC) and liquid chromatography (LC), detection systems such as mass spectrometry (MS), ICP-OES, nuclear magnetic resonance spectrometry (NMR), ultra violet (UV) and visible light spectroscopy and enzyme based assays (Tretheway et al. 1999, Fiehn *et al*. 2000, Sumner *et a*l. 2003, Weckwerth 2003, Kopka *et al* 2004, Desbrosses *et al*. 2005, Froes *et al*. 2009). The metabolome of primary compounds such as amino acids, sugars, organic acids and others can be measured by conventional GC-MS after derivatisation to increase the volatility of the molecules.

The aim of this study was to investigate the nutrient content and metabolite profiles of selected native and exotic plants associated with *Wiseana*. In particular, silicon, carbon, nitrogen, carbon-nitrogen ratio, acid detergent and neutral detergent fibre were considered to support the possibility that the choice of an insects host plant is actually linked to the plants nutritional status. It is hypothesized that plant nutrient content and metabolite profiles play key roles in porina preference for exotic pastures. This knowledge is crucial because it might explain why porina have become successful in New Zealand pastures and also provide useful information for their management.

6.3 Materials and methods

6.3.1 Plant species

Plants for this study are the same plants used in Chapter 5.

6.3.2 Sampling for plant nutrient analysis (silicon, carbon, nitrogen, carbon-nitrogen ratio, acid detergent and neutral detergent fibre).

At 24 weeks, six plants per species in their vegetative growth stages (phenological growth stage determined by observation) were selected at random and the whole plant was cut just above the soil surface at noon, assuming peak physiological activity. The shoot of each plant was put in a labelled brown paper bag. The bagged shoots were oven dried at 65℃ (Contherm, ThermoTec 2000) for 24 h. Once dried, each sample was milled using a grinder and passed through a 1 mm sieve, then put in labelled resealable clear plastic bags (15 cm × 8 cm). All milled and sieved plant samples were stored in an airtight plastic container at room temperature. Dried plant powder of single plants was used for the following analyses.

6.3.2.1 Silicon

Silicon (Si) was extracted from the plant material (six replicates per plant) by digesting 0.1 g of ground plant material in 2.5 mL of 50% sodium hydroxide and 3 mL of 30% hydrogen peroxide with 5 drops of an antifoaming agent (octanol), made up to 50 mL with deionized water and autoclaved at 120℃ for 60 min (Snyder 2001). The digest was analysed for Si using the Varian 720 ICP-OES (Inductively Coupled Plasma Optical Emission Spectrophotometer) (Varian, Australia) with standard setting (axial torch power of 1.20 kW, plasma gas flow 15.0 L/min, aux 1.5 L/min and nebulizer 0.9 L/min). The concentration of Si in the sample was related to the intensity of lines in its optical spectrum. The emitted light was spectrally resolved by diffractive optics, and the intensity of light was measured with a detector (Nölte 2003), with complete wavelength coverage from 167-785 nm and a resolution of 7 rpm to capture all wavelengths in one simultaneous reading. Calibration standards and internal standards were serially diluted from Merck ICP standard solutions using MilliQ water (Barnstead). Calibration curves were generated using at least four standards and a standard blank.

6.3.2.2 Total carbon, nitrogen and carbon-nitrogen ratio

Total carbon (C) and nitrogen (N) were analysed using an Elementar Vario-Max CN Elemental Analyser. Samples (2 mg, six replicate per sample) were combusted at 900℃ in an oxygen atmosphere. The combustion process converts any elemental carbon and nitrogen into CO_2, N_2 and NO_x. The NO_x species were subsequently reduced to N_2. These gases were then passed through a thermal conductivity (TC) cell to determine CO_2 and N_2 concentrations and the %C, %N and C/N ratios calculated from the sample weights.

6.3.2.3 Acid detergent fibre

Acid detergent fibre (ADF) was determined gravimetrically by extraction with a cetyl-trimethylammonium bromide and sulphuric acid solution. An acidified quaternary detergent solution was used to dissolve cell solubles, hemicellulose and soluble minerals leaving a residue of cellulose, lignin and heat damaged protein, plus a portion of cell wall protein and minerals (ash). ADF was determined as the residue remaining after extraction as follows: One gram per sample (six replicates) was dispensed into a 600 mL beaker and the sample weight recorded (sW). Samples were dispensed in duplicate, including one quality control per run. 50 mL of detergent solution (prepared by dissolving 20 g of technical grade cetyltrimethylammonium bromide to 28 mL of 95-98% sulphuric acid and made up to a litre by adding reverse osmosis purified water) was added to each beaker. Beakers were fitted condensers that were brought to boil on a heating apparatus and refluxed for 1 h. When foaming of the sample settled, beakers were swirled and the sides of the beaker rinsed with a small quantity of ADF solution if required. Each sample was poured into a 60 mL Gooch crucible (porosity 1) to collect the fibre residue and the remaining beaker content was rinsed into the crucible with hot water. The detergent was drained using a Büchner apparatus for speed filtration. Samples were not fully drained between rinses to prevent blockage of the crucible. The residue was then washed about six times with hot water until all the detergent was removed and finally rinsed with acetone (Lab grade). Crucibles were placed in an oven and dried at 100℃ (± 5℃) for 12 hours. Subsequently, the crucibles were allowed to cool in a desiccator and weighed, with digested weights recorded as dW. The crucibles were then transferred to a furnace and the content ashed at 500℃ for 2 hours. The cool crucibles were placed in a desiccator and reweighed with sample weights recorded as aW.

ADF was calculated using the following formula;

$$\%\ \mathrm{ADF\ (as\ is)} = 100 \times \left(\frac{(\mathrm{dW} - \mathrm{aW})}{\mathrm{sW}}\right)$$

$$\%\ \mathrm{ADF\ (DM\ basis)} = \left(\frac{\mathrm{ADF}\ \times 100}{\mathrm{rDM}}\right)$$

Where sW = sample weight, dW = digested weight, aW = ashed weight, and rDM = residual dry matter of the sample (determined on an independent sub-sample by standard methods).

6.3.2.4 Neutral detergent fibre

Neutral detergent fibre (NDF) was also determined gravimetrically by extraction with sodium lauryl sulphate, ammonium pentaborate and ethylenediaminetetraacetic acid (EDTA). A neutral

detergent solution was used to dissolve the easily digested pectins and plant cell contents (proteins, sugars and lipids), leaving a fibrous residue (NDF) that is primarily cell wall components of the plant (cellulose, hemicellulose and lignin). The detergent was used to solubilise the proteins and EDTA was used to chelate calcium and remove pectin at boiling temperatures.

One gram per sample (six replicates) was dispensed into 600 mL beakers with sample weights recorded as sW (samples were also dispensed in duplicate, including one quality control per run). 50 mL of detergent solution (prepared by dissolving 19 g of technical grade disodium-EDTA dihydrate, 27 g ammonium pentaborate decahydrate and 30 g of sodium lauryl sulphate in 1 L of reverse osmosis purified water) was added to each beaker. Samples were then refluxed, washed, rinsed, dried and ashed as in 6.2.6 above.

NDF was calculated as follows;

$$\%\ \mathrm{NDF\ (as\ is)} = 100 \times \left(\frac{(\mathrm{dW} - \mathrm{aW})}{\mathrm{sW}}\right)$$

$$\%\ \mathrm{NDF\ (DM\ basis)} = \left(\frac{\mathrm{NDF}\ \times 100}{\mathrm{rDM}}\right)$$

Where sW = sample weight, dW = digested weight, aW = ashed weight, and rDM = residual dry matter of the sample (determined on an independent sub-sample by standard methods).

6.3.3 Sampling for metabolite profiling

Five plant samples per species were selected at random and a 7 cm section cut from the base of each plant (sampling was done at midday because most metabolites are subject to strong diurnal rhythms and are at their peak at midday (Lisec 2006). The samples were sealed in aluminium foil then snap frozen in liquid nitrogen, lyophilised in a freeze dryer (Thermo Savant) for 24 h, and stored at 80°C.

6.3.3.1 Plant metabolite and derivatization

About 40 mg each of freeze-dried sample was weighed (Sartorius CP 2245) into labelled FastPrep vials (MPBiomedicals, USA) containing 2.5 mm zirconia beads, and 1 mL of methanol (80% v/v) was added (without the plant material thawing). A blank was also prepared without the plant material. To each sample, 60 µL ribitol (0.2 mg/ml) was added as internal standard and vortexed for 30 s. Samples were homogenised in an MP FastPrep-24™ (MPBiomedicals, USA) at 4 m/s for 40 s, and centrifuged for 5 min at 20817 *g*. The resultant supernatant was transferred to Eppendorf tubes.

Samples were dried in the Speed Vac (Labconco CentriVap®, USA) at 30℃ for 360 min. Nitrogen gas was added to prevent oxidation and samples were stored in an airtight container at -80℃.

Trimethylsilylation is a commonly used method to derivatise a broad range of metabolites, including sugars, sugar alcohols, amines, amino acids and organic acids, in order for them to become volatile and thermally stable (Roessner *et al*. 2000). A total of 40 µL of methoxyamination reagent (prepared by dissolving 20 mg/mL of methoxyamine hydrochloride in pure pyridine at 20-25℃ in a glass vial) was added to samples, including a blank (empty tube) used as a control, using a glass syringe. The mixture was vortexed and put in a hot-water bath at 37℃ for 90 min. A total of 40 µL of MSTFA reagent (N-methyl-N-(trimethylsilyl) trifluoroacetamide) was then added, centrifuged at 20817 *g* for 5 s and the supernatant was transferred into glass vials suitable for gas chromatograph-mass spectrometer (GC-MS) analysis.

6.3.3.2 GC-MS metabolite profiles

Prepared metabolite samples were analysed using a Shimadzu GCMS-QP2010 Ultra (Shimadzu, Japan) gas chromatograph-mass spectrometer fitted with a Restek Rtx-5ms fused silica capillary column (30.0 m x 0.25 mm i.d. x 0.25 µm, Bellefonte, PA, USA) and supplied with a 5 m guard column. A CTC-Combi PAL autosampler (PAL LHX-xt) was used to inject 1 µL of sample into the GC injection port, operating in split mode at 250℃ and 78.6 kPa pressure at a ratio of 20:1. After injection, the column oven was held at 80℃ for 3 min, then heated to 330℃ at 5℃/min, and held at this temperature for 13 min. Helium was used as the carrier gas with the constant linear velocity set at 34.1 cm/s in split mode (1.0 mL min^{-1}). The mass spectrometer (MS) was operated in electron impact ionisation mode with 70 eV and mass range of 50 to 600 m/z. The temperature of the capillary interface was 250℃, with the source temperature set at 200℃.

6.3.3.3 Mass-spectral tags and their identification

The gas chromatography separates complex mixtures of metabolite derivatives into a series of compounds that enter the mass spectrometer and are subsequently ionized, fragmented and detected. Each metabolite represented by one or more ionic fragments of precise mass, referred to as mass-spectral tags (MST) (Desbrosses *et al*. 2005), with each MST having properties that facilitate unequivocal identification of the parent metabolite (using the linear retention index (IR)), following comparison to a pure reference compound (Wagner *et al*. 2003). MassFinder 4 software was used to visualise and interpret MST tags, by identifying peaks and assigning names to these peaks by matching MST and retention index to reference compounds in the NIST EPA/NIH Mass Spectral Library database (National Institute of Standards and Technology, NIST11) and Wiley

Registry of Mass Spectral Data 10th edition (John Wiley & Sons, Hoboken, New Jersey). Compounds were quantified by calculating their retention indices as follows;

$$\frac{(\mathrm{Rtx}-\mathrm{Rtcv})}{(\mathrm{Rtcn}-\mathrm{Rtcv})}\times 100+\mathrm{RIcv}$$

Where Rtx = retention time of the compound, Rtcv = retention time of the n-alkane before x, Rtcn = retention time of n-alkane after x, RIcv = the retention index of the n-alkane before x.

Further identification of metabolites were done using the Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/). Compounds were quantified if they appeared in at least three of five plant samples.

6.3.3.4 Confirmation of compounds using pure samples

A concentration of 0.2 mg μL^{-1} solution of commercially available reference compounds was prepared by dissolving them in methanol. 60 µL of each sample was transferred into Eppendorf tubes and dried in the Speed Vac at 30℃ for 90 min. A total of 40 µL of fresh methoxyamine was added to samples with a syringe. The mixture was vortexed and put in a hot-water bath at 37℃ for 90 min. A total of 40 µL of BSTFA reagent (N. O-Bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane) was added, vortexed and put in the hot-water bath at 37℃ for 30 min, centrifuged at 20817 G for 5 sec, and the supernatant transferred into glass vials suitable for GC-MS analysis. Samples were run using the same conditions as above. Peaks of the chromatogram of pure samples were then compared with the peaks and mass spectra of identified plant compounds.

6.4 Statistical analyses

Differences between means for the variables Si, C, C/N ratio, ADF and NDF were determined by ANOVA, assuming normality of distribution and homogeneity of variance using Genstat 64-bit Release 18.1, VSN International Ltd. The significant differences between means was tested with Tukey's Honestly Significant Difference test (HSD) at the significance level of P = 0.05.

Multivariate analyses using MVSP 3 (Kovach Computing Services, UK) of the data set was applied as a second, complementary approach to view how well the exotic and native host plants were separated. Hierarchical cluster analysis (HCA) was used to identify similarities in metabolite profiles among host's plants, while PCA was used to explore hidden patterns among host plants where relationships between metabolite and grouping were still unclear. PCA uses a n-dimensional vector approach to separate samples by the cumulative correlation of all metabolite data. This then identifies the vector that yields the greatest separation between samples.

A one-way multivariate analysis of variance (MANOVA) was conducted to test for differences in the groups of chemical compounds in the host plants using Genstat. The significant differences between means was tested with Tukey's Honestly Significant Difference test (HSD) at the significance level of P = 0.05.

6.5 RESULTS

6.5.1 Silica, carbon, nitrogen, C/N ratio, acid detergent fibre and neutral detergent fibre.

Significant differences between means were detected for Si between host plants ($F_{6,35}$ = 48.94, p < 0.001). Si content was the least abundant in *P. tenax*, a native host plant with a mean content of 0.57 g kg^{-1} and most abundant in the exotic host plant, *L. perenne* × *L. multiflorum* with a mean Si content of 16.66 g kg^{-1} (Table 6.2).

Carbon content was significantly different between the putative host plants ($F_{6,35}$ = 224.26, p < 0.001). The C content was lowest in the exotic host plants, *L. perenne* × *L. multiflorum* and *T. repens* (≤ 41%) and higher in the native host plants (≥ 43%, Table 6.2). There were significant differences in N in the host plants ($F_{6,35}$ = 692.17, p < 0.001), with the native host plants low in N (≤ 1%) compared to the exotic host plants (≥ 4%, Table 6.2). Similarly, C:N was significantly different ($F_{6,35}$ = 75.09, p < 0.001) being very low in the exotic host plants (≤ 8%) compared to the native host plants (≥ 24%, Table 6.2).

The ADF content of host plants was also significantly different ($F_{6,35}$ = 2224.46, p < 0.001) with the exotic hosts showing lower in ADF content than the natives; *T. repens* had the least with 18%, and *P. tenax* the most with 43% (Table 6.2). Similarly, NDF content was significantly different ($F_{6,35}$ = 604.22, p < 0.001) with the exotic host plants being characterized by lower NDF values compared to the native host plants; the lowest amount, i.e. 22% was found in white clover and the highest in red tussock (70%)(Table 6.2).

A principal component analysis of the native plants shows distinct discrimination among the five plant species (Figure 6.1), with the first four principal components explaining 97% of total variability. The first principal component (PC1) explained 78% of total variability. Examination of PC1 loadings suggested that the difference between the exotic (*T. repens* (TR) and *L. perenne* × *L. multiflorum* (LO)) and native hosts plants (*P. cita* (PO), *A. squarrosa* (AS), *P. tenax* (PT), *C. rubra* (CR) and *F. actae* (FA)) involved NDF and C on the positive side, and ADF and C:N on the negative side. The second principal component (PC2), explained 15% of total variability. On examination of the

PC2 loadings, this difference between the exotic and the native host plants involved Si on the positive side and N on the negative side.

Further PCA analyses on the native host plants identified differences in their constituents with the first four principal components explaining 86% of the total variability. The first principal component (PC1), explained 63% of total variability, clearly separating the native host plants. Examination of PC1 loadings (Figure 6.2) suggested that the difference between *P. cita*, *A. squarrosa* and *P. tenax* involved C, ADF and C:N on the positive side. The second principal component (PC2), explaining 23% of total variability, separated the native host plants. Examination of PC2 loadings suggested that this difference between *C. rubra* and *F. actae* involved NDF and Si, on the positive side, and N on the negative side. It was expected that *C. rubra*, *F. actae* and *P. cita* from the Poaceae would have similar contents compared to *A. squarrosa* from Apiaceae and *P. tenax* from Asphodelaceae. However, this was only true for *C. rubra* and *F. actae*.

Table 6.2: Mean (± S.E.) silica, nitrogen, carbon, C/N ratio and fibre (ADF & NDF) content in exotic and native host plants (*n* = 6).

Plant species	Si (g kg^{-1})	C %	N %	C: N	ADF %	NDF %
T. repens	1.71(0.08)^{ab}	41(0.04)^{a}	5(0.03$^{)a}$	7.77(0.03)^{a}	18(0.29)^{a}	22(1.28)^{a}
L. perenne × *L. multiflorum*	16.66(0.05)^{d}	41(0.02)^{a}	4(0.01)^{b}	9.32(0.02)^{a}	20(0.16)^{b}	33(1.19)^{b}
F. actae	5.41(0.0)^{b}	45(0.27)^{c}	2(0.15)^{c}	23.92(2.40)^{b}	34(0.09)^{c}	65(0.19)^{d}
C. rubra	10.39(0.04)^{c}	44(0.03)^{c}	2(0.01)^{d}	27.55(0.13)^{b}	34(0.24)^{c}	70(0.17)^{e}
P. cita	4.26(0.09)^{ab}	45(0.06)^{d}	1(0.02)^{e}	37.13(0.54)^{c}	37(0.17)^{d}	65(0.43)^{d}
A. squarrosa	0.99(0.07)^{a}	43(0.15)^{b}	1(0.02)^{e}	37.10(0.57)^{c}	39(0.27)^{e}	50(0.57)^{c}
P. tenax	0.57(0.09)^{a}	45(0.16)^{e}	1(0.08)^{e}	41.17(3.26)^{c}	43(0.06)^{f}	62(0.4)^{d}

Means within a column followed by the same letter are not significantly different (Tukey's HSD, α = 0.05).

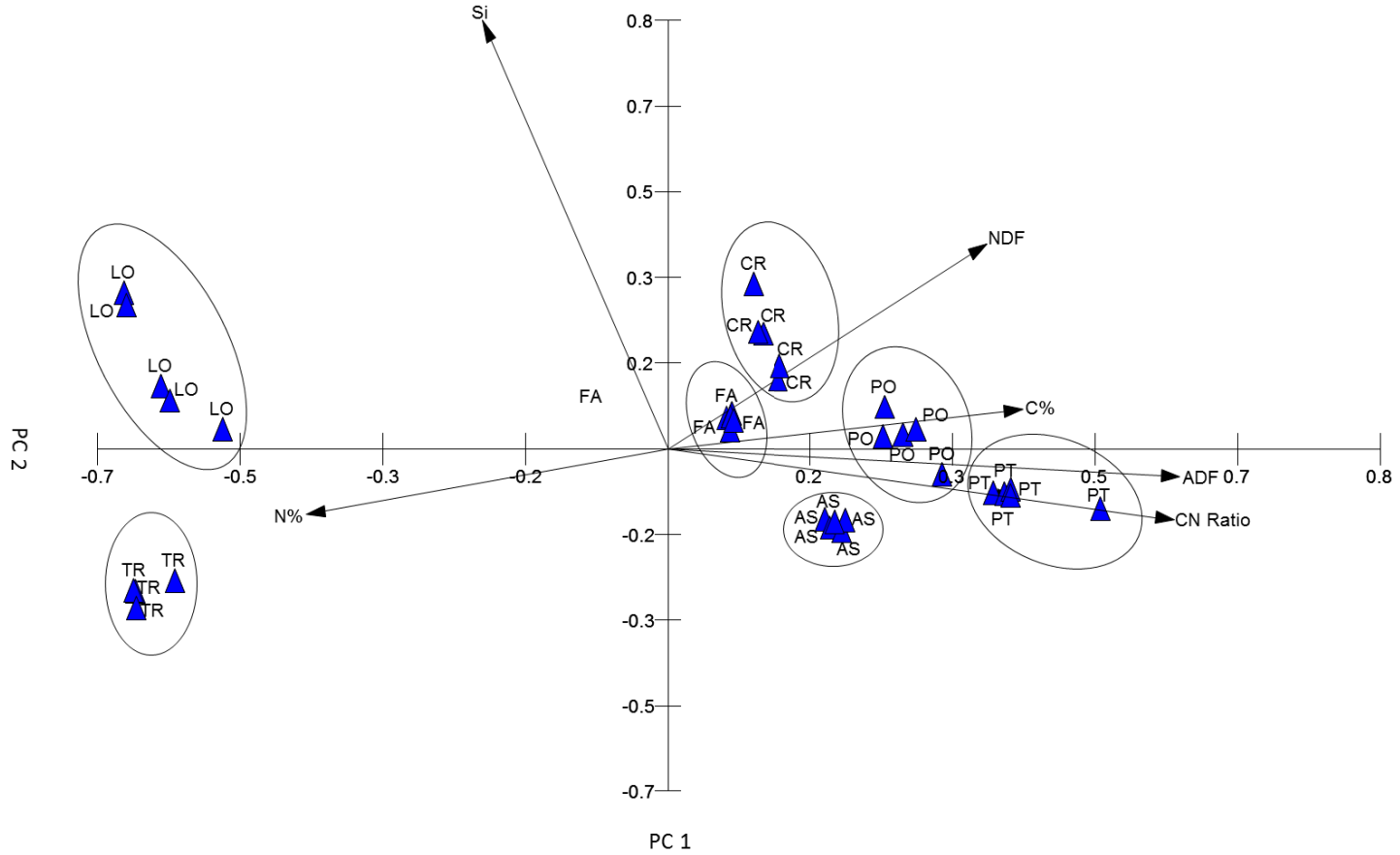
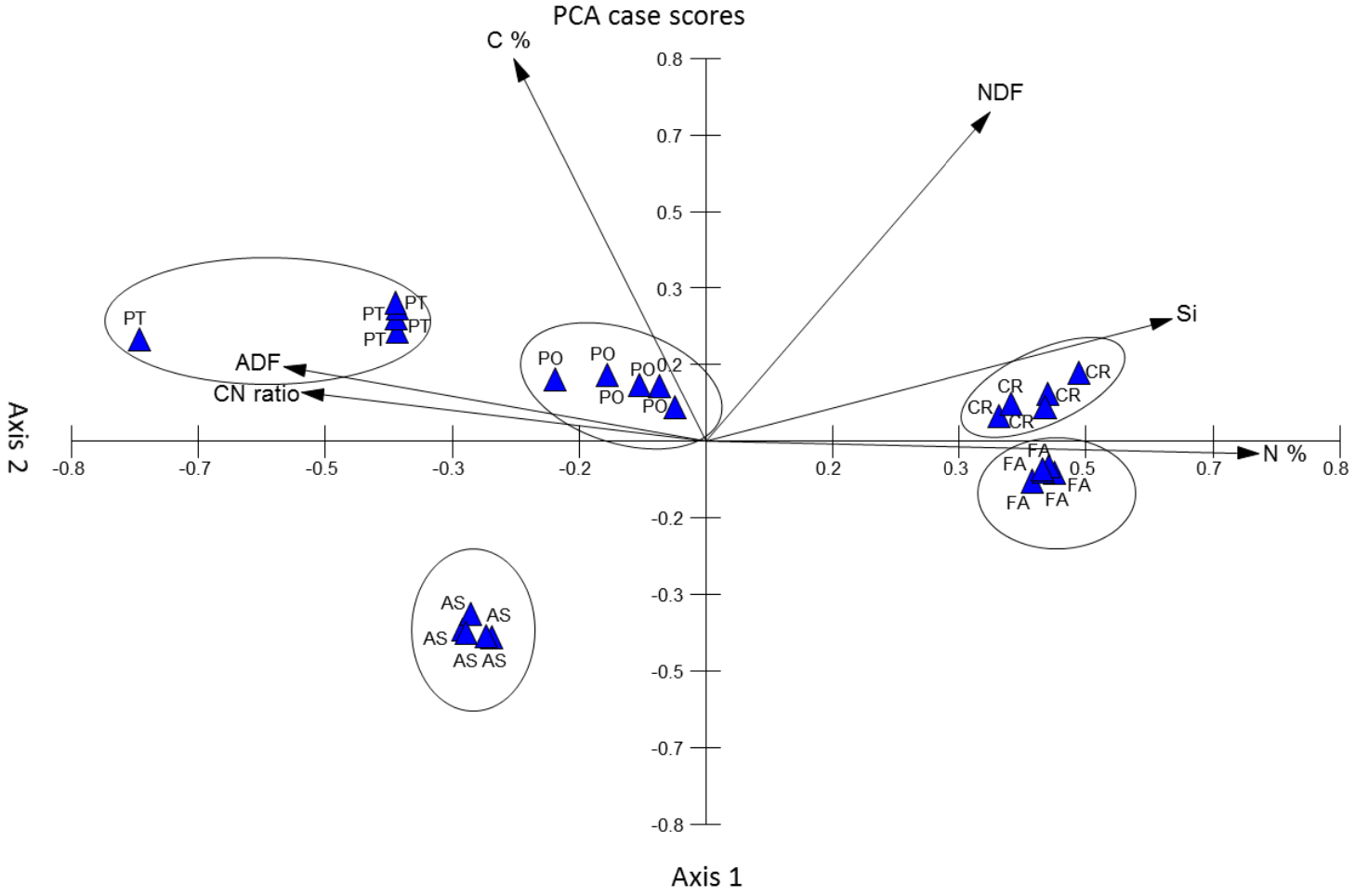


Figure 6.1: Biplot of principal component (PC) analysis of ADF, NDF, N, C, C:N ratio and Si of samples from all seven hosts plants (FA = *F. actae*, CR = *C. rubra*, PO = *P. cita*, PT = *P. tenax*, AS = *A. squarrosa*, TR = *T. repens*, LO = *L. perenne* × *L. multiflorum*). PC 1 = 79%, PC 2 = 15%.



Vector scaling: 1.15

Figure 6.2: Biplot of principal component (PC) analysis of ADF, NDF, N, C, C:N ratio and Si for the five natives hosts (FA = *F. actae*, CR = *C. rubra*, PO = *P. cita*, PT = *P. tenax*, AS = *A. squarrosa*). PC 1 = 63%, PC 2 = 23%.

6.5.2 Primary metabolome profiles of the plants

GC-MS measurements of all seven host plants allowed for the identification of 148 different metabolites categorised into eleven groups. They correspond to 18 amino acids, five cyclitols, three fatty acids, 20 organic acids, three phytosterols, 20 sugars, four sugar acids, six sugar alcohols, eight other N-compounds, seven miscellaneous compounds and 54 unknown compounds (see Appendix 1). The matrix containing the data of these metabolites in the plant hosts was first explored using hierarchical cluster analysis (HCA). Samples clustered together according to species, except for one sample of *T. repens* which clustered with *P. cita* and one sample of *P. cita*, which clustered with *T. tenax* (Figure 6.3).

In the PCA the first four principal components only explained 50% of total variability. The first principal component (PC1), explained 15% of total variability, while the second principal component explained 13%. The PCA of the metabolite did not show any clear separation of the plant hosts. An examination of PC1 loadings suggested some separation of *T*. *repens*, *C*. *rubra* and *F*. *actae* (with an outlier), but *P.cita*, *P. tenax*, *A*. *squarrosa* and *L. perenne* × *L. multiflorum* all clustered together towards the bottom right corner of the biplot (Figure 6.4). Further PCA analyses of the data based on the eleven groupings also did not show any clear separation of the host plants (see Appendix 2).

Some compounds were found to be unique to each host: ***A. squarrosa*** - norvaline, urea, malic acid-2-methyl, phenylalanine, mannitol, galactitol, glucoheptose, melezitose, gibberellin A3, unknown compounds 3, 7 and 49; ***C. rubra*** - aconitic acid, kolic acid, hexadecanoic acid, glucose 6 phosphate, uridine, melibose, unknown compounds 5, 6, 20, 21, 26 and 54; ***P. tenax*** - benzamidine, diethanolamine, hexanoic acid, synephrine, unknown compounds 4, 27, 35, 36, 37, 38, 39, 41, 44, 45, and 50; ***P. cita*** - propargylalcohol, pyroglutamic acid, maleic acid, ribonic acid, otadecanoic acid, unknown compounds 18, 22, 24, 25, 31 and 42; ***F. actae*** - caffeic acid, unknown compounds 14, 28 and 51; ***L. perenne* × *L. multiflorum*** - dehydroascorbic acid and unknown compound 2; and ***T. repens*** with dihydroxymalonic acid, xylose, ononitol, pinitol, unknown compounds 8, 11, 12, 23, 34, 46 and 48.

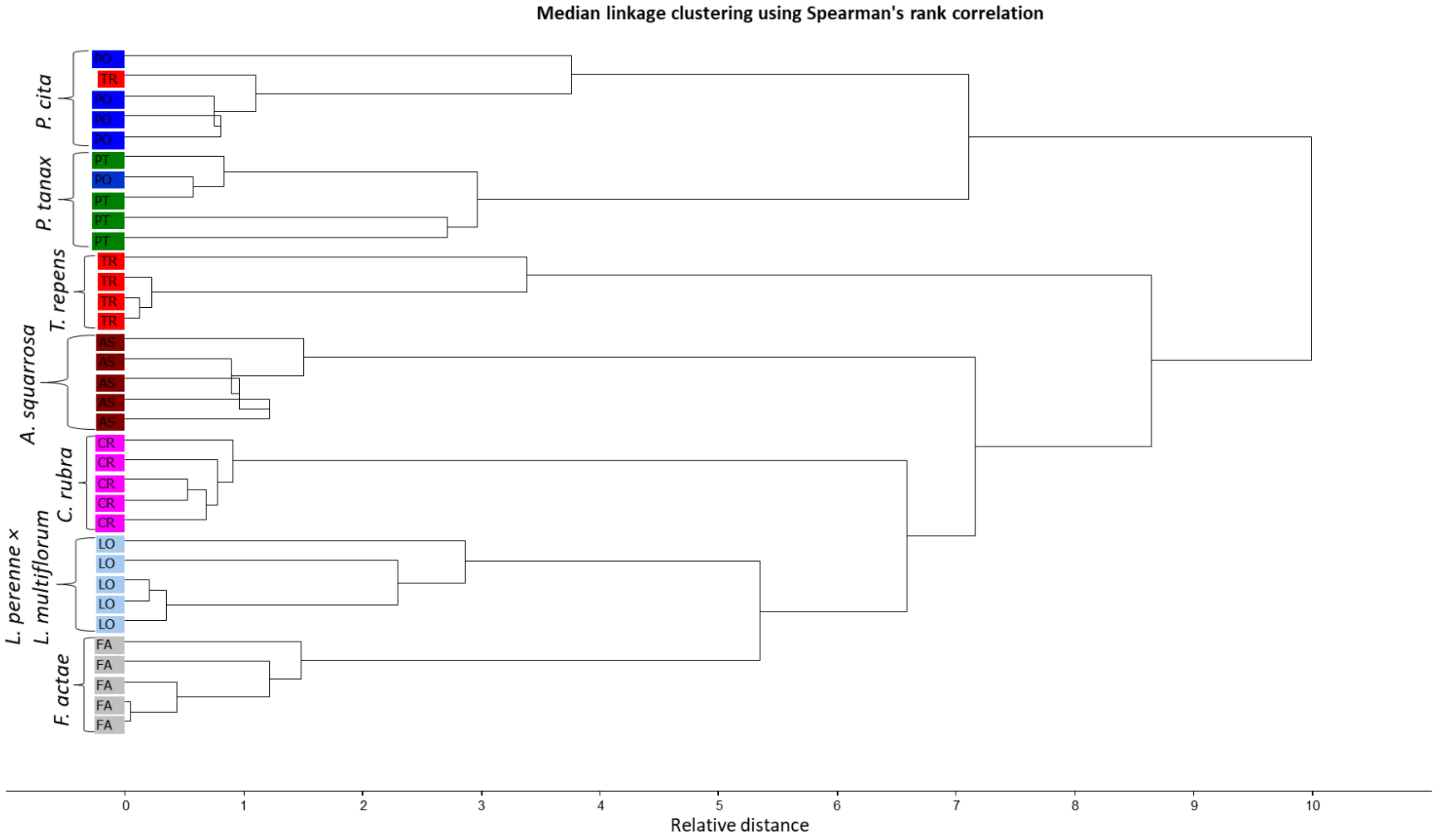


Figure 6.3: Dendrogram of hierarchical clustering of the seven host plants (FA = *F. actae*, CR = *C. rubra*, PO = *P. cita*, PT = *P. tenax*, AS = *A. squarrosa*, TR = *T. repens*, LO = *L. perenne* × *L. multiflorum*) based on metabolite profiles.

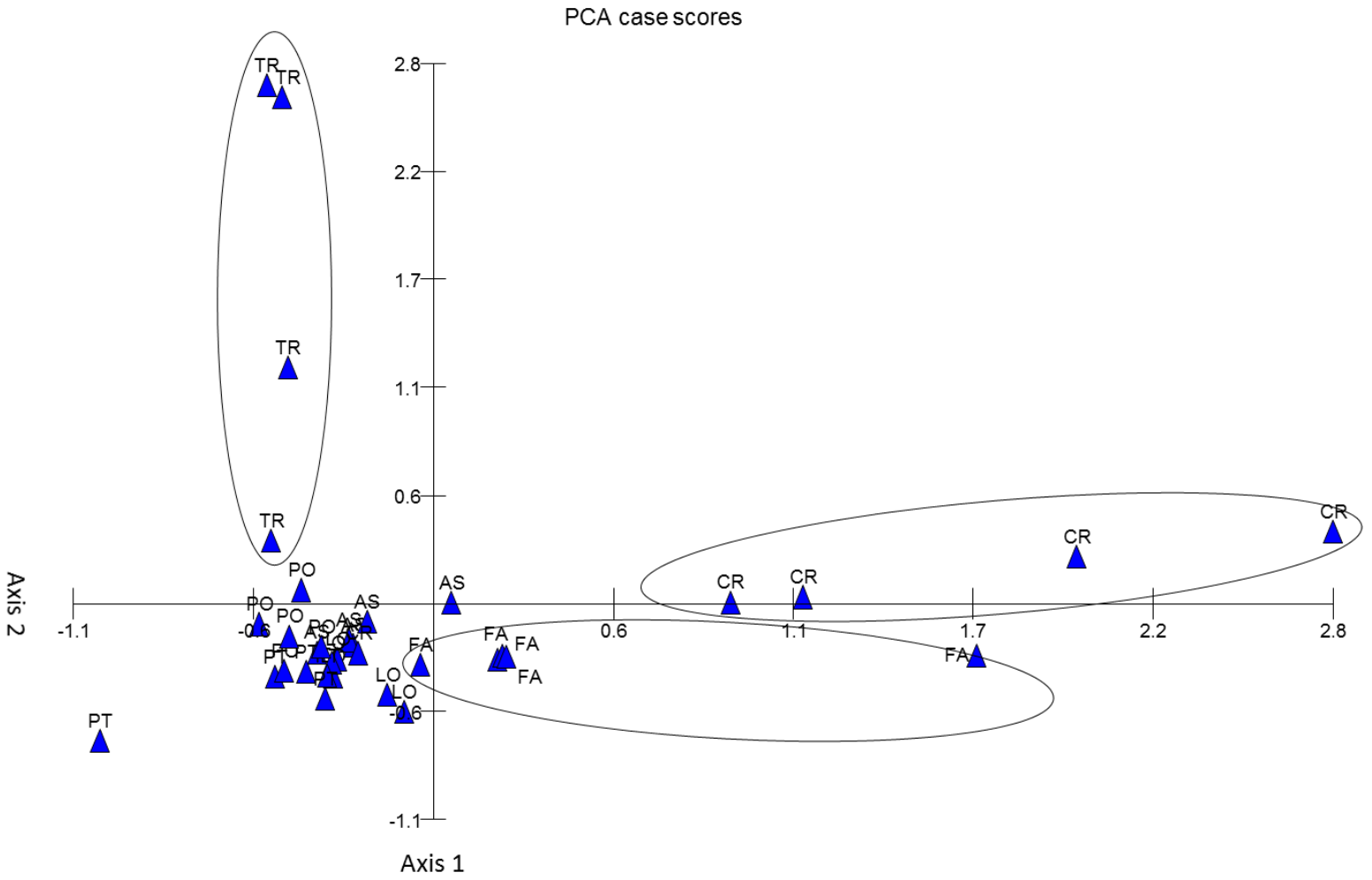


Figure 6.4: Biplot of principal component analysis case scores of host plants metabolite. Percentage of eigenvalues: Axis 1 = 16.6, Axis 2 = 12.8.

The MANOVA showed a statistically significant difference in the host plants for the chemical compound groups, Wilks' Lambda = 0.0024, $F(6, 96) = 0.000$, $p < 0.05$. A series of one-way ANOVAs on each of the eleven dependent variables was conducted as a follow-up test to the MANOVA and the result is presented in Table 6.3, with eight of the variables statistically significant. Post-hoc analyses (Tukey's HSD) were performed to examine differences in the compound groupings across all seven plants. The results revealed significant differences in cyclitols between *T. repens, C. rubra*, and *P. tenax*. Fatty acids in *C. rubra* was different from *A. squarrosa* and *L. perenne × L. multiflorum*, while *C. rubra* was different from *T. repens* and *P. tenax* in sugar content.

Table 6.3: Univariate ANOVAs of chemical classes in the host plants. Means (± S.E.) are given in mg g^{-1} dry weight.

Compounds	*T. repens*	*L. perenne* × *L. multiflorum*	*F. actae*	*C. rubra*	*P. cita*	*A. squarrosa*	*P. tenax*	*F*	*P*
Amino acids	45.02(23.62)^{a}	3.68(1.66)^{a}	136.85(77.91)^{a}	176.00(92.41)^{a}	19.97(6.99)^{a}	17.82(4.26)^{a}	39.89(21.73)^{a}	1.92	0.114
Cyclitols	70.94(19.66)^{b}	14.70(7.72)^{a}	29.47(11.84)^{ab}	3.02(0.91)^{a}	23.56(6.62)^{ab}	0.54(3.32)^{a}	32.10(17.17)^{ab}	3.92	0.006
Fatty acids	0.46(0.19)^{a}	0 (0)^{a}	3.82(2.22)^{ab}	8.47(2.15)^{b}	3.59(1.54)^{ab}	0.32(0.09)^{a}	0.68(0.20)^{a}	5.27	0.001
Miscellaneous	0(0)^{a}	0.81(0.32)^{ab}	6.35(3.77)^{abc}	28.83(12.03)^{ac}	6.17(3.55)^{abc}	14.22(3.67)^{abc}	25.06(8.69)^{abc}	3.30	0.014
Phytosterols	3.13(1.07)^{a}	7.31(5.81)^{a}	2.55(0.51)^{a}	4.60(1.46)^{a}	1.73(0.50)^{a}	1.23(0.17)^{a}	2.77(0.76)^{a}	0.76	0.610
Sugars	239.54(83.73)^{ab}	461.58(149.89)^{b}	399.06(73.55)^{ab}	46.84(11.49)^{a}	393.97(98.24)^{ab}	99.84(20.40)^{ab}	433.11(120.88)^{ab}	3.35	0.013
Sugar acids	1.51(0.60)^{a}	0(0)^{a}	0.52(0.23)^{ab}	0.22(0.06)^{ab}	0.16(0.07)^{ab}	0.40(0.07)^{ab}	1.03(0.61)^{a}	2.66	0.037
Sugar alcohol	8.60(2.65)^{a}	11.02(4.89)^{a}	3.82(1.56)^{a}	12.86(5.96)^{a}	14.31(5.15)^{ab}	39.46(7.41)^{b}	18.18(8.68)^{ab}	3.96	0.006
Organic acids	118.41(43.09)^{a}	87.37(36.11)^{a}	69.74(16.45)^{a}	75.55(26.54)^{a}	71.04(19.21)^{a}	4.01(9.27)^{a}	89.52(39.43)^{a}	0.64	0.695
Other N-compounds	2.20(0.89)^{a}	0.93(0.51)^{a}	2.03(0.72)^{a}	4.70(1.75)^{ab}	0.82(0.52)^{a}	0.98(0.22)^{a}	13.97(5.78)^{b}	3.97	0.006
Unknown compounds	14.73(4.21)^{a}	21.44(11.09)^{ab}	29.93(6.54)^{ab}	12.88(4.64)^{a}	16.19(5.41)^{a}	14.24(4.72)^{a}	73.29(26.93)^{b}	3.21	0.016

Means within a row followed by the same letter are not significantly different (Tukey's HSD, $\alpha = 0.05$)

6.6 Discussion

Decades of research have established the importance of plant nutritive and defensive traits for herbivore performance and population dynamics (Awmack & Leather 2002). In a recent assessment of the effects of within population plant trait variance on herbivore performance, using 457 performance datasets from 53 species of insect herbivores, Wetzel *et al* (2016) found that plants might contribute to the suppression of herbivore populations, not only through low average nutritional quality, but also through heterogeneity in nutrient levels. However, studies on native herbivores and the nutritive traits of their native and exotic host(s) are still uncommon. This study investigated the interspecific variation in the nutrient content and metabolite profiles of some putative native and exotic host plants associated with porina.

Not only did the plants differ in their Si content, there were differences between the natives and the exotics. This suggest that a high Si concentration in the shoot is not a general feature of monocot species. However, a phylogenetic variation exists in the Si composition of plants at the family and genus level (Hodson *et al* 2005), as seen with *L. perenne* × *L. multiflorum*, *C*. *rubra*, *F. actae*, *P*. *cita*, *A*. *squarrosa* and *P*. *tenax*. *Lolium perenne* × *Lolium multiflorum* was high in Si, consistent with it being bred for high herbage quality to increase resistance to insect herbivores (Charlton & Stewart 1999, Reynolds *et al*. 2016). The deposition of Si in plant tissues in the form of phytoliths serves as physical defence, which is associated with resistance to insect pests (McNaughton & Tarrants 1983, Katz 2015) and phytoliths deposition increases plant rigidity and physical toughness (Massey *et al*. 2007a). Thus, resulting in the wear down of the mandibles of insects herbivores (Kvedaras *et al*. 2009, Massey & Hartley 2009, Jeer *et al*. 2017), reducing plant digestibility (Massey *et al*. 2006, Massey & Hartley 2006, Frew *et al*. 2016b), and adversely impacting their growth and consumption (Frew *et al*. 2018). Furthermore, heavily attacked plants typically accumulate more Si (Massey *et al*. 2007b, Hartley & DeGabriel 2016), which may partly explain the high Si content in *L. perenne* × *L. multiflorum*. In legumes, Si is important for the promotion of nodule formation (Epstein 2001), which may also explain the amount of Si found in *T*. *repens*.

C and N are essential nutrients necessary for the development and functioning of plants. Plants use carbon dioxide (CO_2) during photosynthesis, the process whereby the plant converts the energy from the sun into a chemical carbohydrate molecule. While C constitutes the basic structure of plants and accounts for 50% of plant biomass (Elser *et al*. 2000) as polymeric cellulose. The native host plants were higher in C (≥ 44%) compared to the exotic host plants (< 41%) and recent evidence suggests that native plants in New Zealand serve as large C sinks (Schwendenmann & Mitchell 2014,

Silvester & Bergin 2017, McNally *et al*. 2017). N correlated negatively with C, but there were differences in the N content of the plants, with the exotics containing higher amounts. *T. repens*, as a legume, has the ability to fix atmospheric N and this probably explains its high N content. New Zealand pastures are mostly sown with *T. repens* and *L. perenne* × *L. multiflorum* to supply high quality feed for animal production (Charlton & Stewart 1999). Nitrogen is a major nutrient for plants and an essential constituent of protein and chlorophyll, it also plays critical roles in various physiological processes (Leghari *et al*. 2016). Every amino acid incorporated into proteins and the levels of proteins in plants are closely correlated to N (Landry & Moureaux 1984). It has been shown that high N content in plants make them more susceptible to insect herbivores (Bernays 1997, Behmer *et al*. 2012). Similarly, the C:N ratios were different between the native and exotic plants, with the natives having very high C:N ratios. Plants with high C:N tend to have increased concentrations of carbon-based metabolites (Lindroth 1996), which are thought to adversely affect the performance of insect herbivores (Whittaker 1999).

Although both ADF and NDF differed amongst the plant species, they were lower in the exotic plants (≤ 20%, 33%) and higher in the natives (≥ 37%, ≥50%) respectively. These two measurements are routinely used in livestock production to determine how much food an animal requires, and how much energy the animal will receive from the consumed food (Carpenter 2017). ADF and NDF together factor into the crude fibre of forages and are a measure of total fibre content and quality in forages which impacts on the productivity and digestion of the animal. While ADF measures the least digestible components in forages, such as cellulose and lignin, NDF measures hemicellulose, cellulose and lignin, the digestible component of the forage. As the ADF increases, the digestibility decreases (Grant 1997), resulting in a decline in forage (plant) quality. When both measurements increase in forage, the quality of the forage declines (Horrocks & Vallentine 1999). This implies that the exotic plants are of good quality, more digestible and high in energy compared to the native plants.

The clear separation of the plants based on their nutrient content (Si, C, N C:N ratios, ADF and NDF) was as a result of the variability in nutrient content between the plants and it was expected that plants from the same family would be similarities in their nutrient content. However, that similarity was only seen between the genera *Festuca* and *Chionochloa* in the poaceae. Why *Poa* is different is not understood.

As expected, the multivariate analysis of primary metabolome data showed that plants species clustered together, showing that individuals of the same species had similar metabolomes However, the clustering of one specimen of *T. repens* and *P. cita* within the *Poa* and *Phormium*

genus respectively may be due to mislabelling of samples during analysis. The separation of clusters of *T*. *repens*, *C*. rubra and *F*. *actae* illustrates the existence of differences between distinct metabolic systems of the others (Roessner *et al*. 2001, Fiehn 2003, Morris *et al*. 2004). While the clustering of *P. tenax*, *P*. *cita*, *A*. *squarrosa* and *L. perenne × L. multiflorum* shows that their metabolite compositions are more similar. A cross-referencing of metabolites among the plants revealed a large number of these metabolites in the categories of amino acid, cyclitol, organic acid and sugars were common to all seven plants, while only a few of the metabolites were common within the fatty acids, phytosterols, sugar acid, sugar alcohol, other N-compound, miscellaneous and the unknown categories in all plants. Common to all plants were the cyclitols such as quinic and shikimic acids connected to the biogenesis of aromatic compounds (Anderson & Wolter 1966), phytosterols such as beta-sitosterol and campesterol regulate membrane fluidity and permeability (Valitova *et al*. 2016), and fatty acids such as hexadecanoic acid and octadecanoic acid which are precursors of jasmonic acid which is associated with plant defence (Creelman *et al*. 1992, Pena-Cortes *et al*. 1993, Weber *et al*. 1997).

Amino acids like serine, proline, and leucine play many critical roles in plants (Hildebrandt *et al*. 2015), acting as signalling molecules (Häusler *et al*. 2014, Ros *et al.* 2014). While glycine and glutamic acid, essential for chlorophyll synthesis and tissue formation (Hildebrandt *et al*. 2015), were also common to all plants. The metabolite group of erythritol, a sugar alcohol found in only *A*. *squarrosa* and *P*. *tenax,* function as an osmoprotectants, antioxidants, and carbon-storage molecules within the plants (Williamson *et al*. 2002, Patel & Williamson 2016). Some metabolites were found to be unique to some plant species, for example, *P*. *tenax* contains benzamidine, a reversible competitive inhibitor of trypsin, trypsin-like enzymes and serine proteases important for defence (Oliveira *et al*. 2014). Gibberellin A3, a plant hormone that stimulates growth and development (Ramwant & Chakrabarty 2013) was found in *A*. *squarrosa*. In general plants produce a diverse array of metabolites as chemical barriers against herbivores (Nishida 2014) or serve as cues for insect herbivory (Honda 1990, Feeny 1992, Ono *et al*. 2000) that may be common to most plants, a few plants or unique to a single plant genus or species.

The group comparisons of compounds showed that the plants had similar contents of amino acids, phytosterols and organic acids as expected, but were different in their contents of cyclitols, fatty acids, miscellaneous compounds, sugars, sugar acids, sugar alcohol other N-compounds and unknown compounds. In a few cases a group of compounds were totally lacking in some plants, such as the fatty acids and sugar acids that were absent in *L. perenne × L. multiflorum*. Sugars are essential substrates in carbon and energy metabolism and in polymer biosynthesis, sugars have

important hormone-like functions as primary messengers in signal transduction (Rolland *et al*. 2002). A high level of sugars (high sugar resistance) in plant tissues enhances plant resistance (Morkunas & Ratajczak 2014). Fatty acids are an important source of reserve energy and essential components of membrane lipids in all living organisms. In plants, fatty acids metabolic pathways play significant roles in pathogen defense (Kachroo & Kachroo 2009).

This study showed that known exotic and putative native host plants of porina contain different levels of Si, C, N, C:N ratio, ADF and NDF, and synthesise a wide range of metabolites that together are essential for growth and development, regulation of important physiological processes and for defence against herbivory. The metabolites and the quantity of these metabolites found in the plants varies from the family to species level. Whether a plant is successfully colonised depends on an interplay between the nutritive qualities and defensive traits of the plant. How the nutrient and metabolite contents of these native hosts affect the development and fitness of porina is discussed in Chapter 11.

Chapter 7

Does the foraging behaviour of the *Wiseana* species differ: comparing *W. copularis* with *W. cervinata* larvae?

7.1 Abstract

Understanding the foraging behaviour of porina larvae is crucial to effectively manage and control this insect pest in New Zealand pastures. The nocturnal larval foraging behaviours of *W. cervinata* and *W. copularis* were investigated in the laboratory using videos recorded under infra-red conditions. Foraging behaviours were characterised, and videos analysed using BORIS software showed species differences in the number of tunnels constructed, foraging behaviour and foraging sequences. The implication of the observed differences in behaviour is discussed.

Key words: Larval tunnels, *W. cervinata*, *W. copularis*, foraging behaviour.

7.2 Introduction

The ghost or swift moths belong to the Hepialidae, the largest family in the lepidopteran superfamily Hepialoidea with over 500 species described worldwide. (Nielsen *et al*. 2000, Dugdale 1994). They are considered to be among the most primitive lineages of the Lepidoptera and span the continents of Africa, Asia, Australia, North America and South America. Adult moths vary in size with wingspans of between 3.5 cm to 15 cm, with many species known to be swift fliers (Dugdale 1994). Their larvae are concealed feeders, fashioning tunnels in root and stem tissues, moss, decaying wood, litter, or soil. Some ground dwellers can even construct canopies of silk and debris beneath which they can shelter (Grehan 1989). Larvae may feed on roots, stems or leaves (Powell 1980, Tindale 1981, Mitter & Brooks 1983, Rawlins 1984). In New Zealand the moths are found in most vegetation types including forest, shrubland, grassland, tundra, swamp and bog (Tindale 1938, 1981, Dumbleton 1966, Dugdale 1975), with a focus of biodiversity in the southern South Island, particularly in native forests and in high-country shrub/grassland and swampy habitats (Dugdale 1994).

It has been challenging to study how porina larvae forage because they are nocturnal and live in burrows. Only a few studies have been done on *Wiseana* larvae that focused on their feeding behaviour with a variable degree of success. In a study of invaded stand mixtures, Harris (1969) observed porina larvae first, re-formed the artificial burrow followed by a circular feeding zone

around the burrow entrance with larvae feeding on white clover and cocksfoot in preference to ryegrass. Esson (1970) used time-lapse photography to observe porina behaviour in the field, concluding that it was extremely difficult to present any data on preference or sequence of observed behaviour because of the fluctuation in the number and timing of larval emergence and the amount of time spent aboveground. Although, Esson (1970) confirmed that frost inhibited feeding, he noted that his observations of their cyclic feeding pattern should be interpreted with caution because feeding and burrowing ranges of individuals overlapped and the camera failed intermittently. In his study of food intake and feeding behaviour of porina larvae in the field, French (1973) did not observe any feeding preference because larvae were restricted within metal sleeves with the surrounding area bare of vegetation and only fed freshly cut clover leaves placed at the entrance of their burrows. Although he stated that daily aboveground feeding behaviour was irregular, he did not provide any data to support this. Kain *et al*. (1979) modified French's methodology by placing a metal plate with a 1 cm hole at the centre over the burrow entrance on which different plant species and wheat bait were placed at varying distances from the centre. The Kain et al (1979) study showed larvae to be indiscriminate feeders showing no preference between the different foliage types or bait and concluded that the distance from the burrow was the over-riding factor influencing food preference. Pottinger (1980) observed that larvae feed by nipping foliage off in the vicinity of their tunnels, which they extend along the surface of the ground as their feeding range enlarges. French (1981) also noted that feeding behaviour was very irregular with a non-feeding period of 1-10 days. During this period they remain in the soil for about 6-9 months, feeding and growing before pupating (Barlow *et al*. 1986). It is these subterranean larvae which cause pasture damage during autumn, winter and spring, the level of damage depending on the locality and year. It is typical to find two or more species in pastures. The damage to pasture has been exacerbated further by the large-scale transitions of native grasslands or forests and woodlands into pastures (Frew *et al*. 2017).

To effectively manage and control this insect pest in New Zealand pastures, it is essential to understand the aboveground feeding behaviour of the porina larvae because it will provide insight on how damage is done to pasture plants and how porina species feed on the different plant species that make up pasture. These studies focused mainly on the amount eaten by the larvae when presented with cut foliage within a restricted space, with no categorical data on larval feeding behaviour and behaviour duration aboveground. Furthermore, the *Wiseana* species used for these studies are now in doubt. This study presents sequential and quantitative data on the aboveground nocturnal feeding behaviour of *W*. *cervinata* and *W*. *copularis* using real-time video recording of larvae feeding on live plants.

7.3 Material and methods

7.3.1 Plants

Two exotic pasture plant species, white clover (*Trifolium repens*) and hybrid ryegrass (*Lolium perenne* × *multiflorum*, nil endophyte) commonly sown in New Zealand were used. Seeds of ryegrass and white clover were obtained from AgResearch Ltd and the Lincoln University Field Services Centre respectively in Lincoln, New Zealand.

Four-month-old potting mix was used as the growing medium, which consisted of 80% bark, 20%: Osmocote Exact fertiliser (16 N-3.5 P-10 K), horticultural lime, hydroflo and pumice. The potting mix was sieved through a 4 mm mesh sieve to obtain a fine mix, then put into seeding trays. Seeds of white clover and ryegrass were spread lightly over the surface in different trays, and each tray covered with a light layer of the mix and firmed gently. The trays were watered and placed inside the greenhouse on a flat tray containing water to keep the seed trays just moist enough for germination to take place. Germination took 5-9 days, after which seedlings of white clover or ryegrass were gently transplanted into a 0.5-litre growing pot and moved to the nursery. The seedlings were allowed to grow for twelve weeks and watered regularly before use for the experiment.

7.3.2 *Wiseana* larvae

Rearing methods for *W*. *cervinata* and *W copularis* followed those described by Atijegbe *et al*. (2017) (Chapter 4). Briefly, cultures were started by collecting eggs from adult female porina moths that had been light-trapped at the AgResearch farm, Lincoln, New Zealand during the 2016/2017 porina flight season. After egg laying, moths were stored in tubes of 96% ethanol at 15℃ for identification. The preserved adult females were identified using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method developed by Richards *et al*. (2017) (Chapter 3) so that eggs of each female species can be matched with the hatched larvae. Eggs of each female were hatched on filter paper (90 mm diameter) that had been immersed in a solution of 1 mg/L of copper sulphate for 2 minutes and drained on paper towels to remove excess solution before use. The eggs were sealed in a Petri dish with Parafilm® and stored in a constant temperature cabinet (CT) at 15 ℃. On hatching, larvae in each Petri dish were transferred into a 3 L KLIP IT™ container, (23.5 cm × 17 cm × 12 cm, Sistema Plastics, New Zealand) half full with Grade-2 moist horticultural bark (HB) sourced from Intelligro, New Zealand. Larvae of both porina species were reared for eight weeks in a CT cabinet at 15℃, 90% relative humidity with a photoperiod of 12L: 12D and fed carrot (*Daucus carota*) until larvae were large enough to be handled. Once large

enough (~3 cm long) *W. cervinata* and *W. copularis* larvae were transferred individually into 120 mL plastic bottles half filled with moist HB and fed diced carrot for another ten weeks before being used for behavioural observations. This extended rearing period meant the larvae were at their most active feeding stage and were large enough to be seen during observations.

7.3.3 Set-up and recording of behavioural observations

The experiment was conducted in a growth chamber measuring 2.36 m × 1.57 m × 2.11 m (Conviron, Model BDW40) equipped with Elite Agro ceramic metal halide lamp (Model 930, 355W, Philips) canopy with a transparent glass barrier, and an downward airflow distribution system using an additive control to provide ambient CO_2 conditions inside the room. The room temperature was maintained at 15 ℃ (s.d. ±1.49) with a 12L: 12D photoperiod. Photosynthetically active radiation (PAR) at 1 m below the lamp canopy was 500 µmol-2 s-1 (s.d. ± 10 µmol-^{2} s-1) during the 12-hour photoperiod with the relative humidity in the room maintained at 90% (s.d ± 3%). A 1 m × 0.5 m platform was anchored to the side wall of the chamber 1 m from the floor on which the experiment was set up (the arena). Four AXIS Companion IP cameras (AXIS Communication®) with built-in IR illumination connected to an HP Zbook 17 (G4 Mobile Workstation Intel Core i7-6700HQ 2.6GHz 8MB Cache Processor, 16GB RAM, 1TB wireless, Windows 10 Pro 64bit) with AXIS Companion video management software. Each camera was mounted on a retort stand with the clamp securely holding the camera facing downwards, all four cameras on retort stands were then placed on the arena.

Initially, a pilot study was carried out to determine how best to set-up the experiment; such as setting the day/light cycle to coincide with when the camera was recording, setting the infra-red to come on when the light went off, how long recording would run, the size of container to be used, the number of plants in the container, the number of nights of recording for each larva, and whether to record the two porina species separately or together. Finally, it was decided that a 6 L capacity plant pot (22.5 cm top diameter, 16.5 cm base diameter and 17.8 cm depth) was the appropriate size for the camera to effectively zoom and focus to give a good view inside the pot. A 6 hour infra-red video recording was set to commence at 8.00 pm, stopping at 2:00 am to cover the period over which the larvae were known to feed in the field. The recording was carried out over three consecutive nights because in previous studies by Harris (1969) and Esson (1970) it was observed that larvae feed once every 3-10 days. Sixteen larvae, comprising eight *W*. *cervinata* and eight *W*. *copularis,* was determined to be the maximum number of larvae that could be video recorded within the time frame the growth chamber was available.

Before recording commenced, behaviours or actions exhibited by the larvae were identified and defined creating an ethogram of behavioural events (Table 7.1). The behavioural events were categorised into either a 'point event' (instantaneous without a time duration) or 'state event' (non instantaneous with a time duration) before coding and event logging.

Table 7.1 An ethogram of behavioural events exhibited by porina larvae.

Category	Behaviour	Event type	Definition
Aboveground	Emerged P	State	The larva emerges partially from the burrow with one or more abdominal segments remaining inside the burrow
	Emerged F	State	The larva fully emerges from the burrow with no abdominal segments left inside the burrow
	Searching	State	Larva actively searching for food
	Contact	State	Larva comes in contact with a food source
	Feeding C	State	Larva feeding on white clover
	Feeding R	State	Larva feeding on ryegrass
	Dragging	State	Larva dragging food into the burrow
Belowground	Underneath	State	Larva is seen burrowing and moving just below the soil surface
	Maintenance	State	Larva engaged in reformation of the burrow, removing debris or plugging the entrance
	Enters with food	State	Larva enters the burrow with food and remains in the burrow
	Enters without food	State	Larva enters the burrow without food and remains in the burrow
	Tugging	State	Larva actively feeding and pulling on foliage that is still attached to the plant
	Break	Point	Foliage breaks from plant due to the pulling action of the larva

* A point event is defined as instantaneous without a time duration. A state event, has a time duration.

Each 6 L pot was half filled with HB and two plants each of white clover and ryegrass were transplanted carefully from their 0.5 L pots into each pot with the plants arranged perpendicular to each other so that the two ryegrass plants were opposite each other and the two clover plants were also opposite (Figure 7.1). An artificial vertical burrow was made at the centre of the pot by drilling a hole with a 0.5 cm diameter stick 5 cm deep into the media.

Two larvae each of *W. cervinata* and *W. copularis* were selected at random and each allocated at random to one of four 6 L pots; each pot was labelled and placed at random under one of the four cameras. The larvae were placed inside the artificial burrow and the camera adjusted to about 15 cm from the top of each pot and the cameras focussed to obtain high-quality video recordings (Figure 7.1). The four cameras recorded videos simultaneously with the video of each camera stored on the SD card located inside the camera. Videos of each night's recording were retrieved the next day and stored on an external storage device to prevent the camera from deleting a previous video when the next a video was recorded.

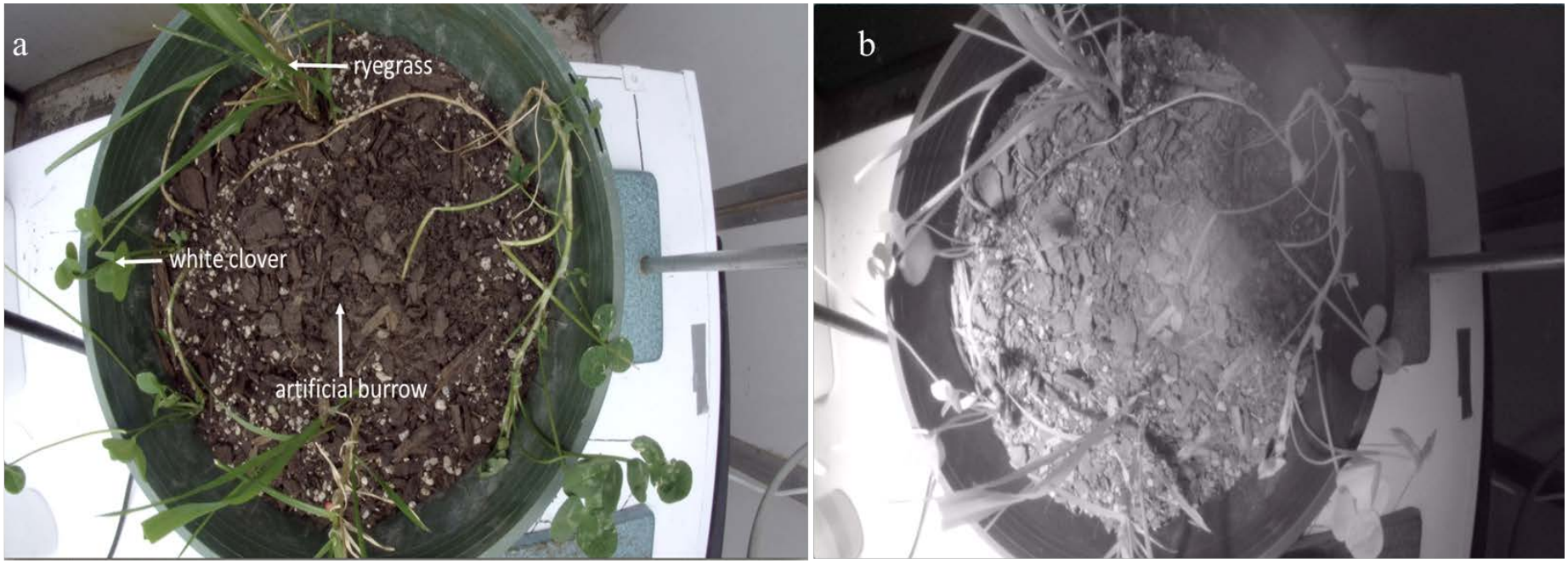


Figure 7.1 a) Camera view of a pot with two plants of white clover and ryegrass under normal light. b) Camera view of a pot under infra-red recording conditions.

After three days of recordings, the four pots containing the larvae were removed, and two new larvae each of *W. cervinata* and *W. copularis* were then selected at random and allocated randomly to one of four new 6 L pots, placed under the cameras, set-up and recorded for three nights. This process was repeated until all eight of the larvae of both *Wiseana* species were recorded.

7.3.4 Video analysis

A total of 48 videos were recorded containing 288 hours of video recordings for all 16 porina larvae. Using the Behavioural Observation Research Interactive Software (BORIS; Friard & Gamba, 2016), each video was viewed in slow mode, and all behavioural events were coded. A time budget of the number and duration of the behavioural events for each coded video was extracted using BORIS. Data on behavioural categories for *W. cervinata* and *W. copularis* were then subjected to statistical analysis.

7.3.5 Statistical analysis

Differences in the duration of each behaviour event between species were analysed using Welch's *t*-test because the data did not meet the assumption of equal variances required for the Student's *t*-test. The Welch's *t*-test is robust enough to handle small and unequal sample sizes, as well as unequal variances while maintaining a nominal Type I error (Ruxton, 2006; Adusah & Brooks, 2011). All statistical analyses were conducted using Sigmaplot 14.0 (Systat Software Inc., https://systatsoftware.com/products/sigmaplot/).

7.4 Results

7.4.1 Tunnelling larval behaviour of *Wiseana*

When the larvae of both species were put inside the artificial vertical tunnel, they were observed to do one of two things: the larvae either reconstructed the tunnel or left the artificial tunnel and created a new vertical tunnel. In either case, the tunnel was lined with silk, resulting in soil upheaval and casts being shed on the soil surface. After that, the *W. cervinata* larvae created a single horizontal burrow from the vertical tunnel to the base of the clover plant where an exit was made close to the plant (Figure 7.2a). The larvae could be seen moving within the horizontal burrow close to the soil surface as a result of their crawling movement disturbing the soil at the surface. Interestingly, *W. copularis* larvae created two horizontal burrows, one each from the vertical burrow leading to the clover plant at opposite ends, with exit holes at the base of the clover plants (Figure 7.2b). A third horizontal burrow was made by two W. *copularis* larvae to the ryegrass host.

W. *cervinata* larvae fed only on clover, while *W*. *copularis* larvae fed mostly on white clover, they also fed occasionally on ryegrass.

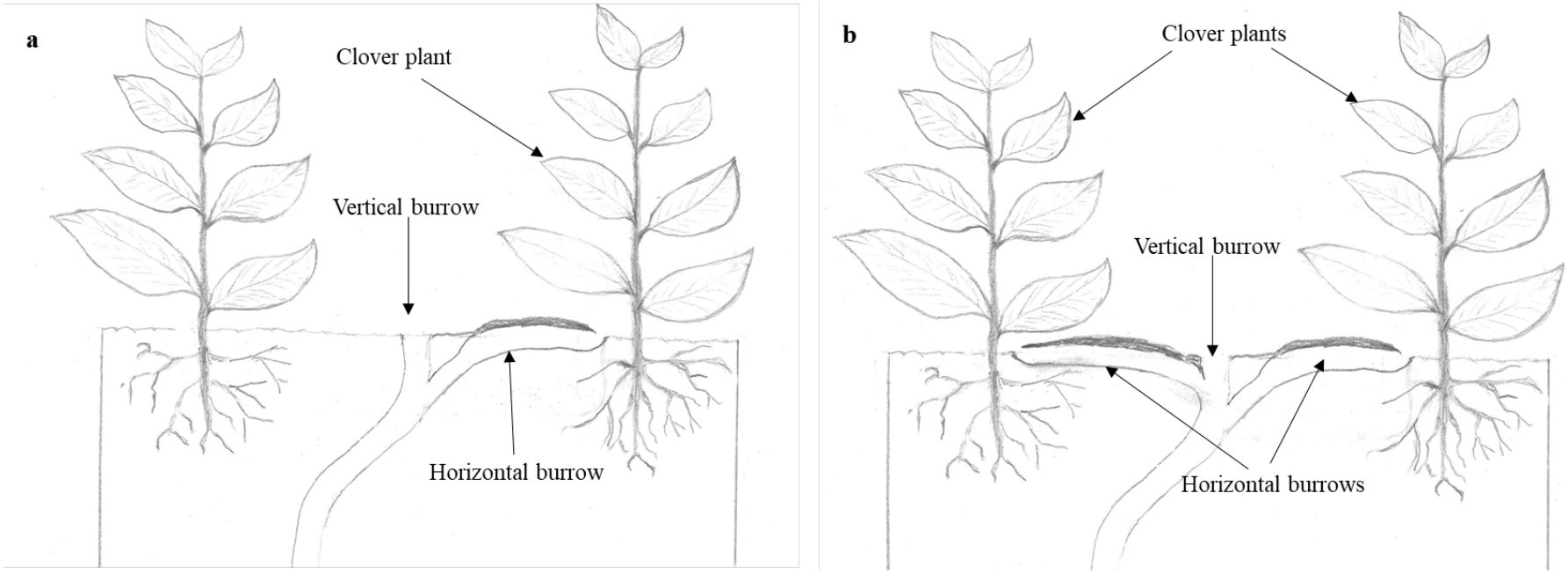


Figure 7.2 Generalised longitudinal sections of subterranean larval tunnels of *Wiseana*, a) *W. cervinata*, b) *W. copularis.*

7.4.2 Foraging behaviour of *Wiseana* species

The results of the analysis of behavioural events are presented in Table 7.2., and the results showed that although *W*. *cervinata* spent more time burrowing just below the soil surface than *W*. *copularis*, however, they were not statistically different. The same applies to the time spent feeding on white clover, dragging food into their burrows and time spent by larvae *W*. *cervinata* entering their burrows with food. Larvae of *W*. *copularis* however, spent more time emerging from their burrows, maintaining their burrows, entering their burrows without food and tugging at the plant, and again statistically they were not different. There was only a significant difference in contact time between the two *Wiseana* species with *W*. *cervinata* spending a long time in contact with the plant before feeding compared to *W*. *copularis*. Although larvae of both species of porina fed on clover when they emerged from their burrows, however, only larvae of *W*. *copularis* were observed to feed on ryegrass. They were observed to feed on stolons, stems and shoots of the host just aboveground level, cutting through with their mandibles and dragging the plant part into the burrow where they stored and then eaten. One larva each of *W*. *cervinata* and *W*. *copularis* emerged fully during the experiment. However, both larvae just crawled around above ground without exhibiting any foraging behaviour.

Table 7.2 Mean duration (minutes) of behavioural events of *W. cervinata* and *W. copularis* larvae (n = 8).

	W. cervinata			*W. copularis*				
Behaviour	**Mean (±se)**	***n***	**Frequency**	**Mean (±se)**	***n***	**Frequency**	***df***	***P* value**
Underneath	128 ± 35	4	8	59 ± 18	5	15	9.12	0.11
Emerged P	4 ± 2	4	14	14 ± 4	5	10	11.87	0.07
Emerged F	0.3	1	1	1.8	1	1	-	-
Maintainance	12 ± 7	4	10	46 ± 21	5	14	7.23	0.16
Searching	5	1	3	4	3	3	-	-
Contact	0.5 ± 0.06	3	8	0.16 ± 0.03	2	5	5.65	0.03
Feeding C	26 ± 13	2	12	22 ± 4	3	8	3.45	0.81
Feeding R	-	-	-	34	1	2	-	-
Dragging	26 ± 22	2	8	21 ± 20	1	3	3.17	0.88
Burrow A	127 ± 57	2	6	18 ± 5	2	5	2.02	0.20
Burrow B	30 ± 24	3	11	39 ± 19	4	14	6.68	0.76
Tugging	9 ± 6	3	9	26 ± 24	2	4	1.11	0.60

7.4.3 Sequences of the total frequencies of above ground larval forging behaviours *Wiseana*

Larvae of *W*. *cervinata* and *W*. *copularis* showed consistent patterns of behaviours with four and five out of eight larvae becoming active, on average 58 min and 60 min respectively, after the night cycle began. Video analysis of aboveground foraging behaviour using BORIS showed that emergence of larvae from the burrow during the experiment was irregular for both species of porina. However, the behavioural sequences once larvae emerged were consistent for *W*. *cervinata* and *W*. *copularis*. Larvae of *W. cervinata* and *W. copularis* became inactive, on average, 16 min and 53 min respectively, before the night cycle ended.

The larvae of *W*. *cervinata* partly emerged from the burrow, searched for the nearby clover plant and on contact they fed. In some instances larvae grabbed hold of the shoot with their mandibles, tugging at it until it broke before dragging it into the burrow (Figure 7.3a) thereby completing the behavioural sequence. The larvae of *W*. *copularis* also exhibited a similar behavioural sequence as *W*. *cervinata* with the exception that *W*. *copularis* fed on both clover plants on separate occasions and one larva fed solely on ryegrass (Figure 7.3b).

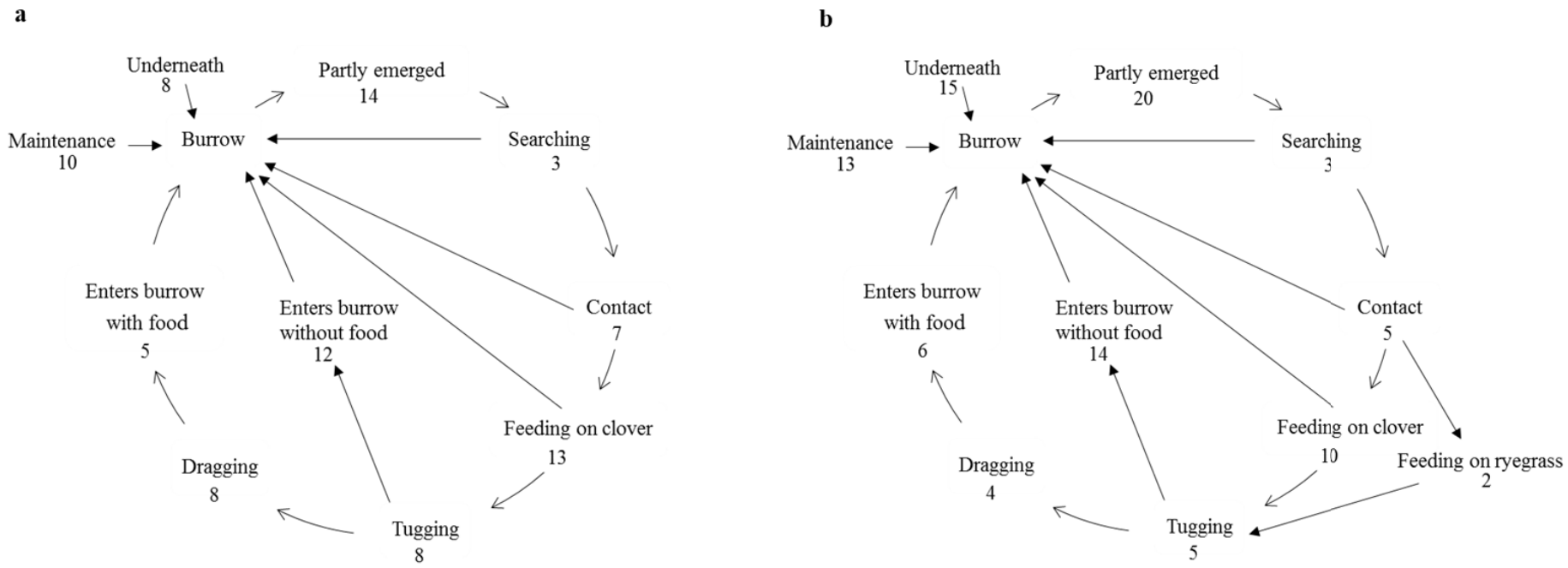


Figure 7.2 Sequence of the total frequency of aboveground larval foraging behaviour for, a) *W*. *cervinata* and b) *W*. *copularis*.

7.5 Discussion

The reconstruction of the artificial vertical burrow or the construction of a new burrow allows the larvae to line inside the burrow with silk giving it some structural integrity to prevent it from collapse. Lining the burrow with silk not only gives the burrow some rigidity, but it also insulates the burrow for the larvae and protecting them from extreme weather conditions. The burrows also shelter them and protect them from predation as they move within the soil in the burrow to the food source. Also within the vertical burrow is located in the storage and feeding chamber, also reported by Greham (1989). The mechanism by which the subterranean *Wiseana* larvae created horizontal burrows and located the plant hosts is not understood but could be in response to some chemical cues emitted by the roots of the hosts. The hypothesis is supported by the review of Johnson & Gregory (2006) on host-plant location and selection by root-feeding insects were they concluded that primary or secondary plant metabolites where the chemical cues that the soil-dwelling insects respond to in the rhizosphere, with CO_2 identified as the principal primary metabolite. Why larvae of *W*. *cervinata* construct a single horizontal burrow while larvae of *W*. *copularis* construct multiple horizontal burrows is not understood, but it does have ecological implications. Having multiple burrows could confer larvae of *W*. *copularis* the advantage of exploiting more of the available food resources and out competing larvae *W*. *cervinata* especially if both species are in the pasture, resulting in more damage caused by *W*. *copularis* larvae. The different emergence times may reduce competition for early stage larvae, but both species are larvae over winter so they will overlap and compete substantially during that time. Perhaps this might partly explain the clear separation in flight patterns between *W*. *cervinata* and *W*. *copularis*. It is possible that *W*. *cervinata* fly earlier so that there eggs hatch earlier and their larvae feed on the host and develop faster before *W*. *copularis* eggs are laid, thus separating the species spatially.

The report by Harris (1969) and Harris & Brock (1972) that larvae feed in a circular manner up to 8 cm radius from their tunnel entrances was not observed. Rather larvae fed at a radius of 2-3 cm removing all palatable herbage around the burrow and they then extend their horizontal burrows to more palatable herbage. It is this foraging behaviour that produces the characteristic circular feeding pattern often seen in the damaged pasture. This is supported by the observations of Barratt *et al*. (1990) who reported that as food supply becomes limited, the larvae move to areas with more food. The study also showed that larvae of both *W*. *cervinata* and *W*. *copularis* fed preferentially on white clover, which agrees with the report of Harris (1969) and Farrell *et al*. (1974) that *Wiseana* larvae selectively grazed on clover. Harris (1969) further attributed the high survival of porina larvae under the mixed stand to the herbage quality of white clover on which they preferentially feed. However, Kain *et al*. (1979) were of the opinion that porina larvae are indiscriminate feeders. Various studies have shown that larvae

eat food offered to them within a restricted space even if it is not suitable (e.g. Farrell 1976, Wagner 1989, Jensen & Popay 2004).

The behaviour of larvae partly emerging to forage is supported by the reports of Esson (1970) and Barrat *et al.* (1999) who observed that *Wiseana* sp. larvae have one or more body segment inside their burrow as they feed for a quick retreat when necessary. Searching helps the larvae to locate their host and on contact, the larvae either fed or dragged it into the burrow after it has been successfully detached from the plant. This behaviour of larvae pulling whole leaf blades still attached to the plant into their burrow was reported by Harris (1969). During the process of tugging and dragging of the food into the burrow, soil particles and debris enters the burrow which is regularly removed by the larvae to keep the burrow clear of any obstruction. The emergence of the larvae of *W. copularis* and *W. cervinata* from their burrow was observed to be inconsistent with larvae from both species not emerging at all for the three consecutive nights during the study. Dumbleton & Dick (1941) and French & Pearson (1981) stated that *Wiseana* larvae in a population could remain inactive for about two to ten consecutive nights probably because of ecdysis. While Esson (1970) attributed the irregular emergence behaviour to the weather extreme conditions. Neither of these observations explain what was observed under the conditions of this observational study. Perhaps the larvae had successfully stored enough food for an extended period without the need to forage for more.

The foraging behaviour and sequence are similar in both species except that larvae of *W*. *copularis* occasionally fed on ryegrass and why the larvae do that is not understood. The study showed that larvae of *W. copularis* spent more time foraging than *W. cervinata* and the amount of time spent being active has a direct relationship with the time spent feeding, which ultimately corresponds with the damage caused by the larvae on pasture.

Although previous attempts have made to study the aboveground foraging behaviour of the *Wiseana* larvae with variable levels of success, this study is the first to successfully characterise these behaviours and describe the sequence of behaviours for two of the species, *W. cervinata* and *W. copularis*. The result of this study showed differences in their tunnel construction, foraging sequences and the amount of time the larvae of both species spent foraging. The single vs multiple tunnel construction is a strong difference, even if there's no way to put a statistical test around it.

Chapter 8

The remarkable locomotory ability of *Wiseana* (Lepidoptera: Heialidae) pupae: an adaptation to predation and environmental conditions?

Results of this chapter is in press

In-Press in *The Weta*. SR Atijegbe, S Mansfield, M Rostás, CM Ferguson and S Worner

8.1 Abstract

The effective management of insect pests requires an understanding of their basic biology and overall life cycle including all life stages. The pupal stage of *Wiseana* spp., a major pasture pest in New Zealand, has been neglected in terms of investigation into its biology for almost a century. This study investigated the locomotory ability of the pupae of two *Wiseana* species (*W. cervinata* and *W. copularis*) inside artificial burrows. Descent time and speed were measured from digital images. Both species descended the tunnel in response to applied stimuli but *W. cervinata* descended slightly faster than *W. copularis*. This is the first study to quantify the movement of a ground-dwelling hepialid pupa inside a burrow.

Key words: Porina, pupae, movement, burrow, *Wiseana*

8.2 Introduction

Wiseana spp. (Lepidoptera: Hepialidae), commonly called porina, are endemic to New Zealand and are found in both North and South Islands (Barratt *et al*. 1990, Dugdale 1994, White 2002). Porina is a complex of seven closely related species (Dugdale 1994, Richards *et al*. 2017) which are major insect pests of pastures, feeding on ryegrass and clover (Harris 1969, Jensen & Popay 2004). Porina have one generation per year and adults fly from spring to autumn with flight times that differ among species, locality and year (French 1973, Carpenter & Wyett 1980, Barratt *et al*. 1990). The larva is the damaging stage of porina and has the characteristic ability to form a subterranean burrow lined with silk (Miller 1971, Zydenbos *et al*. 2011). Burrows are 30-100 cm deep depending on soil type, with the burrow entrance also intertwined with 'silk threads' that hold soil particles together (Barlow et al. 1986). Adult female moths are very fecund (French 1973), with a single female producing between 450-3000 eggs (Stewart 2001, Jackson *et al*. 2012). Depending on temperature and the species involved, eggs will take

about 9-84 days to hatch (French & Pearson 1976, Ferguson & Cook 2004, Atijegbe *et al*. 2017). The small larvae remain in litter on the soil surface for 6-12 weeks (French & Pearson 1981, Barlow 1985, Fleming *et al*. 1986) with the young larvae initially protected by their silken webbing in small crevices on the soil surface (Stewart 2001). The larvae then burrow underground and remain in the soil feeding for about 6-9 months and grow to between 0.75-2g in weight and 6-7 cm in length before pupating outside the burrow (Barlow *et al*. 1986).

As with all insect pests, effective management requires understanding not only of the species' basic biology and overall life cycle, but also details of all its life stages. The pupa is an often-overlooked life stage in many holometabolous insect studies as is true for *Wiseana*. In terms of management this might be because it is assumed that the pupae of most insects are essentially an immobile stage, at most able to wriggle the abdominal segments (Resh & Cardé 2009) and incapable of evading or defending themselves against predators. Hinton (1946) was first to suggest that pupae of ten coleopteran families and three lepidopteran families have a defensive mechanism called "gin traps" that deter predaceous mites. It was Eisner & Eisner (1992) that showed these structures serve as a defensive mechanism to protect coccinellid pupa (*Cycloneda sanguinea*) against predatory ants. Gin traps are essentially mouth-like elaborations of the intersegmental regions of the pupal abdomen where stimulation by a potential predator triggers a flipping response (Eisner & Eisner 1992).

More recent research has demonstrated a wide variety of defence mechanisms used by insect pupae. Mechanosensory mechanisms have been observed in the pupae of the tenebrionid beetle, *Zophobas atratus* (Kurauchi *et al*. 2011, Ichikawa *et al*. 2012, Ichikawa & Sakamoto 2013). The abdominal rotation response by pupae of the tenebrionid beetles, *Tenebrio molitor* and *Z. atratus* functions as an effective defence against larval cannibalism (Ichikawa & Kurauchi 2009). Smedley *et al*. (2002) showed that glandular hairs on a coccinellid pupae (*Subcoccinella vigintiquatuorpunctata*) secrete three polyazamacrolide alkaloids that are potent defences against the predatory ant *Crematogaster lineolata*. Additionally, Alabi *et al*. (2011) showed that cuticular hydrocarbons expressed by *Tribolium brevicornis* pupae have a deterrent feeding effect on both conspecific and congeneric adults, serving as a defence against both cannibalism and predation. Kojima *et al*. (2011) in a novel study of the Japanese rhinoceros beetle (*Trypoxylus dichotoma*), established vibratory communication between larvae and pupae co-inhabiting the same soil where pupae produced vibrations in response to larvae approaching their pupal cells, thus showing anti-predatory and sib-killing-avoidance behaviour.

Significant movement of porina pupae within their burrows had been noted by Dick (1945) and Barratt *et al.* (1990), but details of this behaviour have never been fully described. Porina pre-pupae and pupae are vulnerable in their burrows not only to predators but also to drowning during heavy rainfall over the pupation period of about 30 days. These significant risks for pre-pupae/pupae may have prompted

the evolution of specific behaviours to avoid predation, cannibalism and drowning. Porina larvae do cannibalise one another, but are not known to cannibalise pupae (S. R. Atijegbe, personal observation). Before the introduction by humans of small mammals and exotic birds to New Zealand, porina larvae and pupae were probably preyed on by native birds such as kiwi, pukeko, weka, rock wren, and possibly ground-foraging lizards or carabid beetles. While porina larvae have some ability to defend themselves against predators by rapidly crawling away, 'playing dead'or regurgitating an offensive brown liquid from their mouth (Barratt *et al*. 1990), very little is known about any defence strategy employed by the porina pupae. In this study, the locomotory ability of porina pupae to move up and down their burrows in response to vibrational stimuli was observed and the key morphological features of the pupae that support this movement are described.

8.3 Materials and methods

8.3.1 Insects

Adult female porina moths were hand collected from a light trap located at the AgResearch farm on Springs Road, Lincoln, New Zealand, between 21:00 h and 01:30 h during the flight season (October - March). Individual females were placed into numbered 120 mL PP screw cap plastic bottles (Labserv®) so that offspring could be traced back to their mothers after each female had been identified to species. Moths were kept at room temperature close to a 40-watt light source for about 4 hours and were excited by shaking the bottle once or twice. This procedure encouraged oviposition within 1-2 days. After egg laying, moths were stored in tubes of 96% ethanol at 4°C for identification. The preserved adult females were identified using a molecular method based on the restriction fragment length polymorphism signatures of the mitochondrial cytochrome oxidase I gene (Richards *et al*. 2017).

Eggs of each female were hatched on filter paper (90 mm diameter) that had been immersed in a solution of 1 mg/L of copper sulphate for 2 minutes and drained on paper towels to remove excess solution before use. The eggs were sealed in a Petri dish with Parafilm® and stored in a constant temperature (CT) cabinet at 15°C. On hatching, larvae in each Petri dish were transferred into a 3 L KLIP IT™ container (23.5 cm × 17 cm × 12 cm, Sistema Plastics, New Zealand) half full with Grade-2 moist horticultural bark (HB) sourced from Intelligro, New Zealand. The larvae were reared for eight weeks according to the method described by Atijegbe et al. (2017). After eight weeks, larvae of *W. cervinata* and *W copularis* were transferred individually into 120 mL plastic bottles half filled with moist HB and fed twice weekly with chopped carrot (*Daucus carota*) roots until pupation.

8.3.2 Glass-sided container and artificial burrow for observation

A two-sided glass container, length 27 cm × 5.5 cm × 22.5 cm (Figure 8.11a) was filled with moist HB and compacted. An artificial vertical burrow 20 cm deep was made by pushing a 4 mm diameter stick

down the glass/HB interface to the bottom of the container so that the burrow was visible from the side and pupal behaviour could be observed easily (Figure 8.1b). The pupa was placed at the mouth of the burrow at the beginning of each observation.

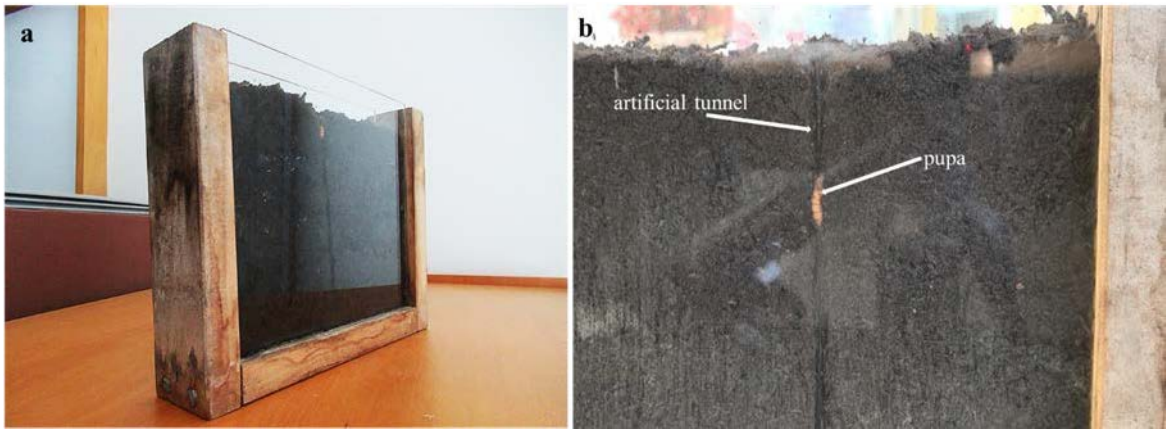


Figure 8.1: (a) Glass-sided container and (b) pupa in the burrow.

8.3.3 Vibrational stimulation

Pupae of *W*. *copularis* (*n* =5) and *W*. *cervinata* (*n* =5) were used for this experiment; sample size was limited due to larval mortality before pupation. A small metal spatula was used to stimulate the larvae by gently tapping the glass and the surface of the moist HB for about 20 s to create vibrational stimuli. Each pupa was observed for about 30 minutes after stimulation.

8.3.4 Images and videos

Behavioural responses of all pupae were captured on video using a camcorder (Sony HDRPJ710V High Definition Handycam 24.1 MP, 10x Optical Zoom, 32 GB Embedded Memory and Built-in Projector). Videos of porina burrow movement were analysed using Behavioural Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016). Digital images of pupal specimens were taken using a Nikon SMZ25 stereomicroscope with a Nikon NIS-Element Imaging Software. Independent *t* tests were conducted to test for any significant differences in descent time and descent speed.

8.4 Results

8.4.1 Pupal movement

The stimulation of *W. copularis* and *W. cervinata* pupae resulted in a vigorous abdominal response inside the burrow. All pupae retreated to the bottom of the burrow by a spiralling motion of the abdomen aided by rows of spines on the dorsal and ventral surface that gripped the burrow wall (Figure 8.2). Each pupa exhibited a series of complex abdominal movements, which consisted of a

couple of sinusoidal wriggling movements followed by an anti-clockwise rotation of the pupa in a corkscrew-like motion which propelled the pupa downwards. Similarly, sinusoidal wriggling of the abdomen followed by a clockwise rotation propelled the pupae upwards. Slow motion observation of the video footage showed that the spines served not only as anchors on the walls of the burrow to provide traction, controlling the speed of pupal descent/ascent, but also gave the corkscrew-like rotation to their movement (https://youtu.be/5raQ_fxs5Go).

The pupae of *W*. *cervinata* descended significantly faster and at a significantly greater speed to the bottom of the burrow than *W*. *copularis* pupae. However, there was no significant difference in the number of rotations between the two species. (Table 8.1).

Table 8.1: Mean (± sd) descent time, descent speed and the number of rotations for pupae of *W*. *copularis* and *W*. *cervinata* (*n* = 5 pupae for each species).

Variables	***W. copularis***	***W. cervinata***	***t***	***p***
Descent time (min)	29.4± 1.5	27.4 ± 1.1	2.36	0.046
Descent speed (cm/min)	0.68 ± 0.04	0.73 ± 0.03	2.32	0.049
Number of rotations	7.8 ± 1.3	7.6 ± 0.5	0.32	0.760

8.4.2 Pupal morphology

Porina pupae of both species were 25 ±1.03 mm long, light brown in colour with a deep brown head capsule (Figure 8.2, only *W. copularis* is illustrated and described). A spiracle was located on either side of each abdominal segment (Figure 8.2a). There are eight abdominal segments and each segment has two rows of spines projecting from ledges that are separated from each other on the dorsal surface of the exoskeleton (Figure 8.2b). On the ventral surface (Figure 8.2c), each segment has a single row of spines. Each segment also bears several very fine elongated setae on the dorsal, lateral and ventral surface of each segment (Figure 8.2d).

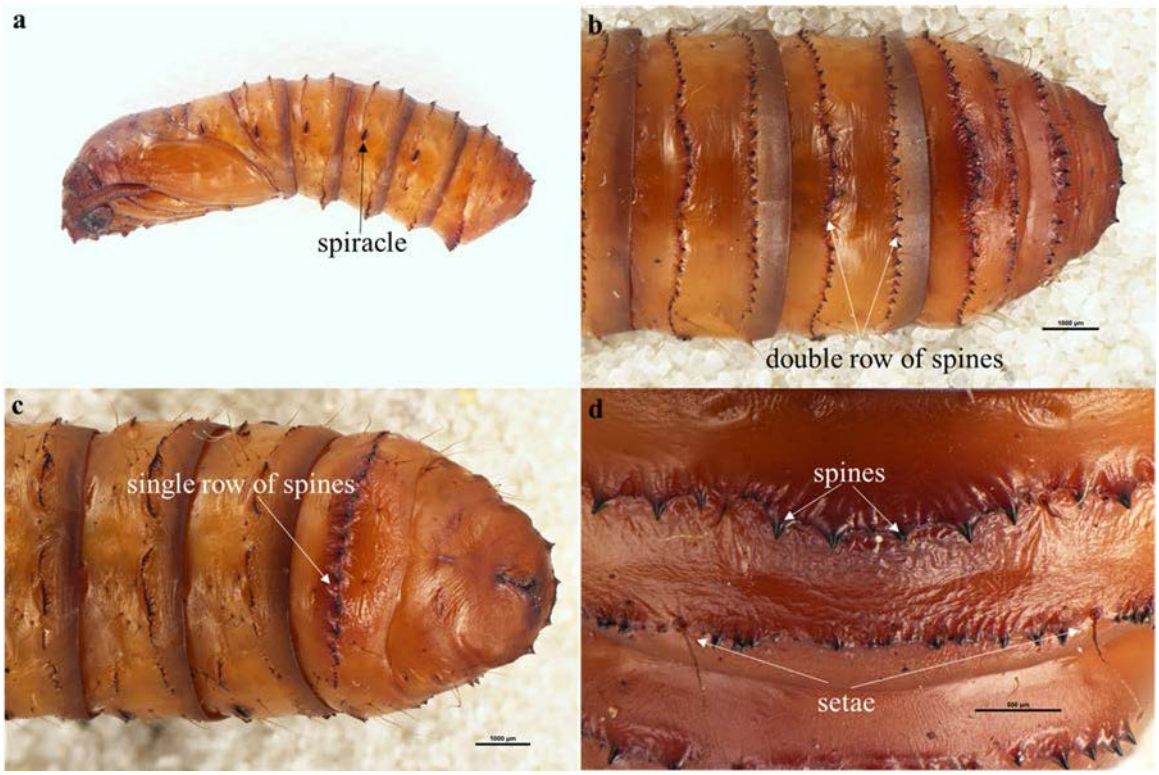


Figure 8.2: (a) *Wiseana copularis* pupa with abdominal segments, (b) dorsal surface, (c) lateral surface and (d) ventral surface.

8.5 Discussion

This study investigated the locomotory ability of *Wiseana* pupae in response to vibrational stimuli in an artificial burrow. Both porina species were responsive to the stimulus, descending into the burrow in a similar way by a vigorous movement of the abdomen and rotation of the pupae, with *W. cervinata* moving slightly faster than *W. copularis*. In the field, larvae secrete silk which is used to line the inner walls and mouth of the burrow; as a result the larvae may be able to move faster than in the artificial burrow. The silk lining strengthens the structural integrity of the burrow, preventing it from collapse or clogging by soil and plant materials. Vibrational stimuli were possibly detected by the *Wiseana* pupae by the setae on the pupal abdomen functioning as mechanoreceptors. Lakes-Harlan *et al*. (1991) suggested that the thoracic and abdominal hairs or bristles act as mechanoreceptors in early insect larvae. This was demonstrated by Kojima *et al*. (2012) in the pupae of *Trypoxylus dichotoma* beetle and recently, Urbaneka & Kapusta (2016) showed that the the dorsolateral setae of *Forcipomyia nigra* are mechanoreceptors.

The abdominal movement by porina pupae after receiving the stimulus consists of sinusoidal wriggling of the abdomen followed by a rotation of the pupa. A similar abdominal movement was observed by Ichekawa *et al.* (2012) in the tenebrionid beetle *Zophobas atratus,* and they hypothesized "that the central nervous system (abdominal ganglion) may possess a neuronal mechanism that generates a motor pattern that rotates the abdomen in one (i.e., clockwise or anticlockwise) direction". It is likely

that pupal mobility is facilitated by pupal body segments connected by soft, intersegmental membranes (Ichikawa & Skamoto 2013). The membranes allow significant abdominal movement generated by periodic pumping activity that increases the hydrostatic pressure in the hemocoel, an activity also crucial for circulation and respiration (Wasserthal 1996, Ichikawa 2009). Additionally, the rows of spines on the abdomen of porina pupae serve as anchors to the burrow wall, providing support and stability to the pupae during movement and preventing them from sliding down the burrow. By providing the pupae with traction when moving up or down the burrow, the spines create the characteristic cork-screw rotational spinning motion observed as they ascend or descend. Pupae use a similar sinusoidal wriggling motion that is aided by the spines to bury themselves when placed on the surface of moist HB (S. R. Atijegbe, pers. obs.).

Under natural conditions, porina pupae remain in the burrow for about 20-40 days until they emerge as teneral imagines (Quail 1900, Dumbleton 1945). That is a significant amount of time over which movement is important for increasing pupal survival under different conditions. Temperature plays a key role in the development of insect pupae, and their ability to move within the burrow is crucial for their survival. Such movement helps temperature regulation by moving to the top of the burrow where the temperature is warmer on sunny days and moving down to avoid freezing conditions at the soil surface during periods of frost or snow in winter (porina overwinter as larvae, often pupating in late winter or early spring). Secondly, this locomotory ability helps the pupae to avoid death by drowning during heavy rain, by allowing pupae to move above the water level in their burrows. Additionally, we hypothesize that such movement plays a role in defence of *Wiseana* pupae against predation from native birds such as kiwi, pukeko, weka and rock wren. When the pupae are located close to the mouth of the burrow, they can move downwards to evade a predator. Finally, such movement is important to place the pupae in an appropriate position for emergence. Dick (1945) observed that pupae wriggle to the surface of their burrows and project half their body length above ground-level before eclosion.

Close examination of *W. copularis* pupae showed key morphological features that may prove to be species-specific (i.e., the number and arrangement of spines and hairs illustrated here). Currently only adults males can be identified from external morphological characters (Dugdale 1994) while all other life stages must be identified by molecular methods (Richards et al. 2017). Pupal characteristics can be used to determine sex in *W. cervinata* (Waller 1964), but have not been described for the entire species complex. Thorough examination of pupal morphology across the different *Wiseana* species may provide new identifying features to complement existing methods.

This is the first attempt to study the pupal movement of *W. cervinata* and *W. copularis*, and it conclusively shows that pupae respond to vibrational stimuli in their burrows.

8.6 Acknowledgements

We thank Richard Townsend for providing the light trap, the late Rob Phiskie for hosting the trap on the AgResearch farm, Myles Mackintosh for providing the glass container and John Marris for help with photos of the pupa. We also thank the New Zealand Aid Programme (NZ Aid), Hellaby Trust, Macmillan Brown Agricultural Scholarship, University of Port Harcourt, Nigeria, and New Zealand Plant Protection Society (NZPPS) for supporting the lead author's research.

Chapter 9

Thermal requirements and degree-days for the egg-larval development of *Wiseana copularis* and *Wiseana cervinata* using linear and nonlinear models.

9.1 Abstract

Two linear models (the standard linear model and the Ikemoto-Takai model) were compared for goodness of fit for the egg-larval development of *W*. *copularis* and *W*. *cervinata*. Other important parameters such as the optimum temperature for development (T_{opt}) and the lethal upper temperature (T_{max}) were estimated by the two non-linear models Briere-1 and the Lactin equation; these nonlinear models were also used to help verify an appropriate value for the most important developmental parameter, T_0. Such parameters along with T_0 indicated the range of temperatures over which life stages might not survive and may be useful for studies involving predicting insect distribution and response to changing climate. The Ikemoto-Takai model gave the highest estimate of T_0 for *W. copularis* at 7.5℃ compared with 6.4℃ for the standard linear model. The Briere-1 and Lactin equation gave estimates of T_0 as 6.3℃ and 6.7℃, respectively. For *W*. *cervinata* the estimates were very similar ranging from 6.8℃ (Ikemoto-Takai model), to 6.9℃ (Lactin equation), 7.0℃ Briere-1 to 7.1℃ (standard linear model). Of the two linear models, the Ikemoto-Takai model had the better fit and lowest standard error for the parameters of interest. Thus, the Ikemoto-Takai model was chosen as the best model to provide the critical parameters, T_0 and degree-days (DD) for egg-larval development of *W. copularis* (7.5℃, 205 DD) and *W. cervinata* (6.8℃, 167 DD) that were used for the design of phenology models to improve their control in New Zealand pastures**.**

Key words: linear model, nonlinear model, degree-day, *W*. *copularis*, *W*. *cervinata*

9.2 Introduction

The use of temperature-dependent developmental data and their application in insect population models have long been recognised as a critical tool to help solve pest problems (Ardab *et al*. 2016). For example, insect phenological models have historically been used to help insect pest management programs to predict seasonal emergence, density, and survival of insect pests under field conditions, and to further help farm managers and entomologists optimise their control, i.e maximise the economic margin gained when investing resources to reduce insect numbers through the use of the

appropriate control strategy (Herrera *et al*. 2005). These models range from simple linear models (Campbell *et al*. 1974) to non-linear models that involve mathematical equations of varying complexity (Worner 1992).

Linear models are widely used to describe development rates of insects in relation to temperature. The linear model is easy to implement and has an added advantage of providing the basis of physiological time calculations by means of two derived parameters: the lower threshold for development (T_0) and the thermal constant (often referred to as the number of accumulated degree days (DD) or accumulated degrees above the lower temperature threshold, T_0, required to complete development of a particular stage of interest. These parameters can then be used to develop simple phenological models to predict the timing of life cycle events for target species. Despite the wide use of linear models, it is well known that development rate curves are never fully linear and tend to be curvilinear at extreme temperatures. There are a wide range of nonlinear equations that can be used to describe this. However, nonlinear models are not easy to use in a practical sense for phenology prediction. The complex equations depend on having access to hourly weather data to ensure precise predictions. Such data is often not at hand and accumulated error over time can make them unreliable (Worner 1988). Recently, several studies (e.g. Tran *et al*. 2012) have involved using the development rate observations over the mid temperature range to fit a linear model to calculate the physiological time parameters. They have used non-linear models that estimate parameters that 1) provide a notional or conceptual value of T_0 (Shi *et al*. 2015) to validate the value of the lower threshold derived from the linear model, and 2) calculate the optimum temperature and maximum or lethal upper temperatures for a species. The overall aim in this study was to compare the fit and performance of two potential linear models to choose the best model to estimate the lower threshold for development (T_0) and thermal constant (degree-days) required for egg hatch for two *Wiseana* species that are pests in Canterbury pastures. The objective was to use these parameters to develop a prototype phenology model that may improve forecasting of insect population events and therefore improve the timing of the implementation of control measures for pest *Wiseana* species in Canterbury and possibly elsewhere in New Zealand (Chapter 3).

9.3 Methods

9.3.1 Data

Developmental data for egg hatch of *Wiseana* species, *Wiseana copularis* and *Wiseana cervinata* in relation to constant temperatures are described in Chapter 4 and Atijegbe *e*t *al*. (2017). Those data were combined with larval-pupal development data summarised by Ferguson & Crook (2004) to determine an appropriate model that could be used to estimate developmental parameters and thereby used for phenological forecasting (Table 9.1). An additional data set for *W. copularis*

development at 13°C (C.M. Ferguson pers. comm.) was added to this dataset to increase the sample size. While the studies were carried out a number of years apart, they used similar experimental conditions, and it is sensible to use all the relevant information at hand to fit and compare models.

Table 9.1: Development of *W*. *copularis* and *W*. *cervinata* eggs.

	W. copularis		***W. cervinata***	
Temp.	**Days**	**Dev. rate**	**Days**	**Dev. rate**
10[1]	84	0.012	45	0.022
13[1]	33	0.030	27	0.037
13[1]	31	0.032	-	-
15[2]	32	0.031	16	0.063
16[1]	19	0.053	24	0.042
20[1]	18	0.056	15	0.067
22[2]	17	0.059	9	0.111

[1] Additional data provided by Ferguson & Crook (2004). [2] See Chapter 4

9.3.2 Linear models

The standard linear model and Ikemoto-Takai linear model (Ikemoto & Takai 2000) as well as two non-linear models were compared for their goodness of fit and performance.

(a) The simple standard linear model

This has been often used to model the relationship between insect development rate and temperature. In this study, a linear model was fitted to the data that fell within the linear portion of the development rate curve (Figures 9.1 and 9.2), to estimate a lower threshold for development (T_0) and the thermal constant in degree days (DD). A linear model was chosen because both T_0 and the thermal constant derived from the linear development rate model are widely used for insect phenology prediction (Worner 1992). Other considerations for using the linear model as a parsimonious approach to modelling species phenology for practical application were 1) both linear and non-linear development rate models can be sensitive to the temperature variability and the profile of a particular site, and neither approach has been adequately shown to outperform the other under all conditions, especially in oceanic climates (Worner 1988), 2) nonlinear model performance under fluctuating conditions can be variable (Worner 1992), and 3) there is no evidence that a single nonlinear model is better than any other under all circumstances, especially under field conditions.

Estimation of the lower threshold for development and thermal constant was based on linear regressions for egg development over the linear range of *W*. *copularis* and *W*. *cervinata* development

versus temperature. The developmental rate (1/development time in days) versus temperature relationship is described by the linear equation:

$$y = a + bx + \varepsilon \quad (1)$$

Where y is development rate (1/days), *x* is temperature, *a* is the intercept, *b* is the slope and ε the deviations of the data from the equation (errors). The parameter T_0 was estimated by solving the equation for development rate (*y*) = 0 (in other words $T_0 = -a/b$). The DD required for complete development (the thermal constant) was estimated by $1/b$ (Campbell *et al*. 1974, Worner 1988, Nahrung *et al*. 2008). To fit the linear model, observed developmental rates at low and high temperatures were examined to detect nonlinearity to determine if any points needed to be removed because of the sensitivity of the slope parameter to nonlinearity. Fitting a straight line to unconstrained data or a non-linear portion of the curve could result in unrealistic estimates (Lamb 1992, Bergant & Trdan 2006, Walgama & Zalucki 2006). Even slight nonlinearity around the optimum temperature will result in a slope that can give an inaccurate lower threshold and thermal constant. Nonlinearity in the development rate curve at high temperatures could result in an unrealistic or even negative lower threshold for egg hatch. If data were required to be constrained to the linear portion of the curve, this was done by maximizing the coefficient of determination R^2 that is a measure of the variability accounted for by a straight line. The coefficient of determination is also a measure of the quality of fit of the linear regression to the data (Zar 1999).

Because of the potential effects of non-linearity on the standard linear model, regression results were examined for high leverage or influential observations using two main diagnostics: Leverage and Cooks distance (Altman & Krzywinski 2016). An observation with high leverage will pull the regression line towards it and can often pull the fit away from the other points. This can cause significant changes in the slope of a regression which can significantly influence the calculation of T_0 and DD creating additional uncertainty to the usual measurement error which is important to try to minimise in this study.

For simple linear regression, the leverage is given by:

$$h_i = 1/n + (x_i - \bar{x}\,)^2 / SS_x$$

Where $SS_x = \sum(x_i - \bar{x})^2$ (Cardinali 2013, Altman & Krzywinski 2016)

Leverage is always between 1/n and 1, and a leverage score greater than (2p + 2)/n is considered high where p = the number of predictor variables.

Cooks distance (Cook 1997, Altman & Krzywinski 2016) indicates an influential observation. Values above 4/n, where n is the number of observations could be a problem. Cook's distance is a 'leave-one -out' measurement of how the fitted values (or, equivalently, the slopes) depend on each observation. Cook's distance is

$$\sum (j(\hat{y}_j - \hat{y}_{j(i)}{}^2)/(p+1) \times MSE$$

Where $\hat{y}_{j(i)}$ are the fitted values obtained by excluding observation *i and MSE* is the mean squared error of the regression.

(b) The Ikemoto-Takai model.

The standard linear model may result in a lower T_0 and a larger DD, due to statistical issues associated with nonlinearity in the upper and lower range of temperatures (Ikemoto & Takai 2000, Arbab *et al*. 2016), Ikemoto & Takai (2000) derived an equation from the standard linear model to get more reliable values. The Ikemoto-Takai linear model is:

$$DT = K + tD$$

Where D represents developmental duration (days), T represents constant temperature (℃), K represents the sum of effective temperatures or the thermal constant (DD) and represents the lower developmental threshold (T_0). One should note that the Ikemoto-Takai equation involves a different relationship to all the other models used in this study. Those models describe the relationship between y = 1/*D* on *x*. The Ikemoto-Takai equation involves regressing DT on the development duration. This means model evaluation parameters such as the RMSE and AIC described in following sections have a different scale to the standard linear and non-linear models and therefore cannot be used to compare models.

9.3.3 Nonlinear models

A number of nonlinear models of developmental rate, as a function of temperature, have been proposed over the decades (Stinner *et al*. 1974, Logan *et al*. 1976, Sharpe & DeMichele 1977, Lamb 1992, Lactin *et al*. 1995, Briere *et al*. 1999). These models involve mathematical expressions of varying complexity, but several different models have been reported in recent studies as being useful descriptors of insect development. Two of the more common non-linear models, the Briere -1 (Briere 1999) and the Lactin equation (Lactin 1995) are chosen for study here. Briere (1999) actually proposed two models, but Briere-1 was chosen based on the finding by Shi *et al.* (2015) who, in a study of six non-linear models fitted to 10 temperature-dependent datasets of insect development, determined

the better model for small samples was the Briere-1 model. In addition, the Lactin equation also has been well used in developmental studies (Broufas *et al*. 2007, Wu *et al*. 2009, Shi *et al*. 2015).

Along with the lower threshold for development, the optimum and maximum temperatures for development make up the three cardinal temperatures for the species (Régnière & Logan 2003). Because the optimal and maximal temperatures are useful for modeling both insect phenology and population dynamics, the Briere-1 and Lactin nonlinear models were fitted to the unconstrained data to estimate these parameters for the egg - larval development stages of *W*. *copularis* and *W*. *cervinata*.

The formula for the (Briere (1999) model is:

$$y = ax(x - T_0)(T_{max}\text{-}x)^{1/2}$$

Where x is the rearing temperature (℃), a is an empirical constant, T_0 is the lower development threshold and T_{max} is the lethal temperature threshold. The optimum temperature is calculated by the formula: $T_{opt} = [4\ T_{max} + 3\ T_0 + (16\ T_{max}^2 + 9T_0^2 - 16T_0T_{max})^{1/2}]/10$ (Briere *et al*. 1999, Grout & Stoltz 2007).

The formula for the Lactin (1995) model is:

$$y = e^{px} - e^{[pTm - (Tm - x)/\Delta T]} + \lambda \quad \text{Lactin (1995)}$$

Where x is rearing temperature (℃), p, T_m, ΔT, and λ are fitted parameters. The optimum temperature for development is determined by the formula: $T_{opt} = [\Delta T * \log_e(\Delta T * p)/(1-\Delta T * p)] + Tm$ (Ramalho *et al*. 2009).

The Lactin equation does not have an analytical solution to determine (T_0). However, the intersection of the equation with the x axis provides a notional estimate which can be found by a numerical method to determine the temperature at which the development rate is zero (Shi *et al*. 2015).

9.3.4 Criteria for data and model selection

For the linear models, the coefficient of determination, R^2 from the regression analysis was used as a measure of goodness of fit. The Akaike criterion (AIC) calculated using $AIC = nLn(SSE/n)+2P$ (Haghani *et al*. 2009) where n is the number of observations, P is the number of parameters including the intercept, and SSE (sometimes referred to as RSS) is the residual error, and the root mean squared errors (RMSE) were used to help determine the best model. The RMSE is a measure of the average deviation between data points and the model. Good models have high R^2 low AIC and low RMSE.

The non-linear models were assessed based on the residual error from the nonlinear regression, the RMSE and the Akaike criterion (AIC) (Haghani *et al*. 2009). The best-fitting model was selected based

on a low RMSE (low average deviation between the observed data and the predicted data) (Shi *et al*. 2015) and a low AIC.

$$RMSE = \sqrt{RSS}/(n - p + 1)$$

Where RSS is the residual error, n is sample size and p the number of parameters.

Unfortunately, there is controversy around using the coefficient of determination (R^2) or adjusted R^2, (R^2_{adj}) to evaluate the fit of a non-linear equation (Spiess & Neumeyer 2010). The current literature within many disciplines suggests that this constraint is still not generally known. Therefore, R^2 and R^2_{adj} were retained as assessment criteria for goodness of fit and comparison between all models, but the results are interpreted with caution.

$$R^2 = 1 - (\frac{S_y^2}{S_{td}^2})$$

Where S_y^2 = variance of residuals and S_{td}^2 is he variance of development rates

$$R^2_{adj} = 1 - \frac{n-1}{n-p}(1 - R^2)$$

9.4 Results

9.4.1 Linear models for insect development

(a) The standard linear model

Leverage and Cooks distance measurement for the standard linear models for both species are shown in Table 9.2. The threshold for very high leverage and Cooks D for *W. copularis* is 4/n = 0.57 and 4/n = 0.67 for *W. cervinata*. For both species both leverage and Cooks D are high for the observations at 22℃, but are not above the threshold for either measure of influence for *W*. *copularis*.

Table 9.2: Leverage and Cooks D measures of influence for both species.

	W. copularis		***W. cervinata***	
Temp	**Leverage**	**Cooks D**	**Leverage**	**Cooks D**
10	0.436	0.492	0.534	0.083
13	0.205	0.004	0.259	0.000
13	0.205	0.032	-	-
15	0.146	0.063	0.177	0.084
16	0.145	0.296	0.167	0.140
20	0.328	0.000	0.330	0.374
22	0.534	0.529	0.534	1.363

Cooks D for the development rate of *W. cervinata* eggs at 22℃ is above the threshold indicating that it is an observation that could be troublesome. Additionally, leverage is high but not above the threshold for development at 10℃ as well as 22℃. These results indicate the observations in the high-temperature range need careful study. Figures 9.1a and 9.1b show the observed development rates plotted against temperature for each species.

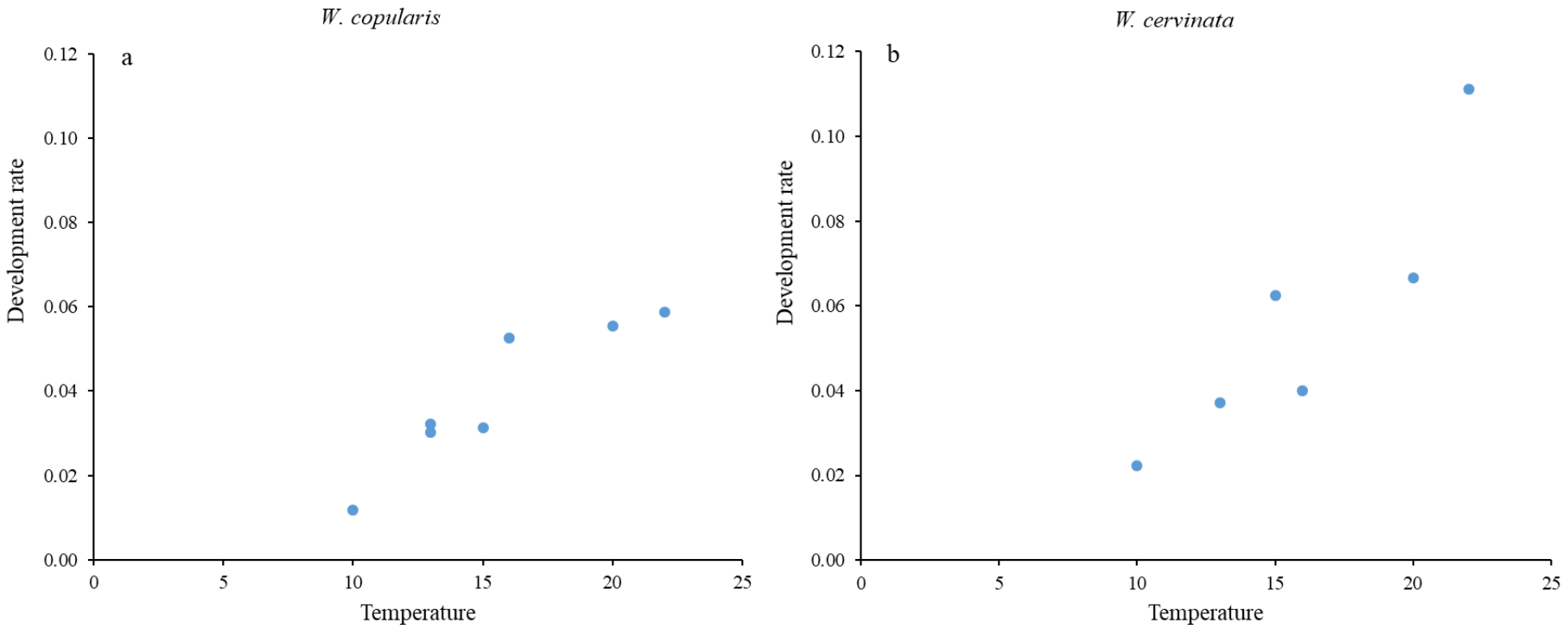


Figure 9.1: Development rates for both species versus temperature using all data observations.

For *W. copularis* there is evidence of slower development at both 20℃ and 22℃, than at the lower temperatures investigated, with only a 1-day difference in development between 20℃ and 22℃. An exploratory analysis showed that retaining the 22℃ observation in the data set resulted in a significant change in slope and lower threshold of development (5.3℃). When the high influence 22℃ observation was removed the lower threshold (T_0) increased to 6.4℃, which is in agreement with the results of all models discussed in the following sections. The R^2 decreased slightly from 0.86 to 0.84 but and the RMSE reduced from 0.0064 to 0.0057 indicating improved prediction. RMSE is an absolute measure of predictive accuracy of the model giving more information, while R^2 is a relative measure of the model fit. For these reasons, the non-inclusion of 22℃ data was justified.

For *W. cervinata* the 22℃ observation was retained as there is no evidence of non-linearity at the higher temperatures. However, the above threshold Cooks D measure for this observation (Table 9.2) does suggest that the accuracy of this data point should be carefully considered here as it is for *W. copularis*. Interestingly, the leverage measured for *W*. *copularis* was low compared to the 0.67 threshold for *W. cervinata*. The regressions for both species are shown in Figure 9.2. The goodness of fit statistics and parameter estimates for the linear models are shown in Tables 9.3 and 9.4.

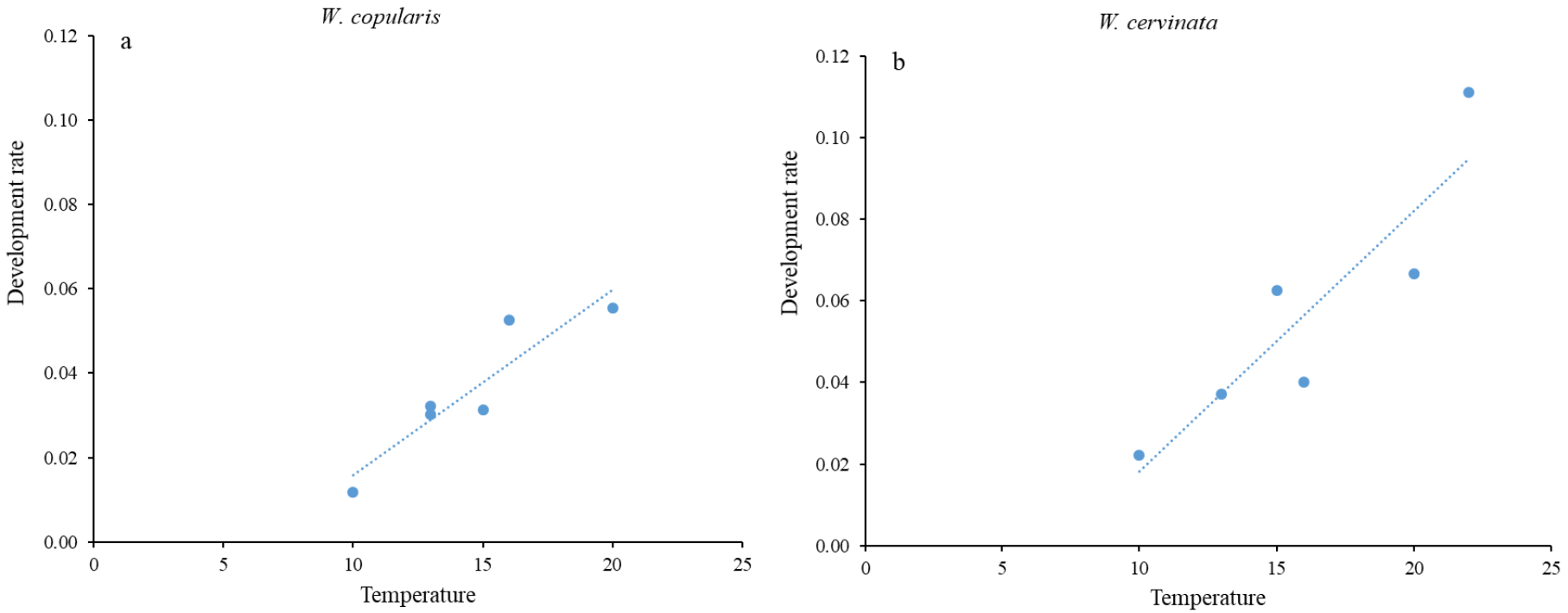


Figure 9.2: Regression lines for *W. copularis* (without the 22℃ observation) and *W. cervinata* using the standard linear model for insect development.

Table 9.3: Fit statistics for both linear and nonlinear models.

Models	Parameter	*W. copularis*	*W. cervinata*
Linear	AIC	-69.3	-48.9
	R^2_{adj}	0.81	0.77
	RMSE	0.0057	0.0015
Ikemoto-Takai linear	R^2	0.97	0.84
	R^2_{adj}	0.95	0.81
	RMSE*	34.2	36.3
Briere-1	AIC	-68.72	-42.93
	R^2	0.97	0.88
	R^2_{adj}	0.95	0.82
	RMSE	0.0057	0.0021
Lactin	AIC	-65.82	-40.34
	R^2	0.96	0.83
	R^2_{adj}	0.93	0.59
	RMSE	0.0068	0.0025

*RMSE of the Ikemoto-Takai linear model has a different scale and should not be compared with RMSE of the other three models.

(b) The Ikemoto-Takai model

Re-arrangement of the temperature development data required to fit the Ikemoto-Takai model increased its linearity as evidenced by the R^2 and R^2_{adj} (Table 9.3, Fig. 9.3). The clear linearity meant that all data could be retained for the regression. The R^2 for *W. copularis* was 0.97 (Table 9.3) compared with 0.86 for the standard linear model using all seven data observations. For *W. cervinata* the R^2 for the Ikemoto-Takai regression R^2 was 0.84 compared to 0.81 for the standard linear model using all data points (Fig 9.3, Table 9.3). All goodness of fit statistics and parameter estimates are shown in Tables 9.3 and 9.4. Note that the linear relationship depicted in Figure 9.3 is quite different from those in Figures 9.1 and 9.2. In Figure 9.3, the duration of development x temperature is regressed against the duration of development.

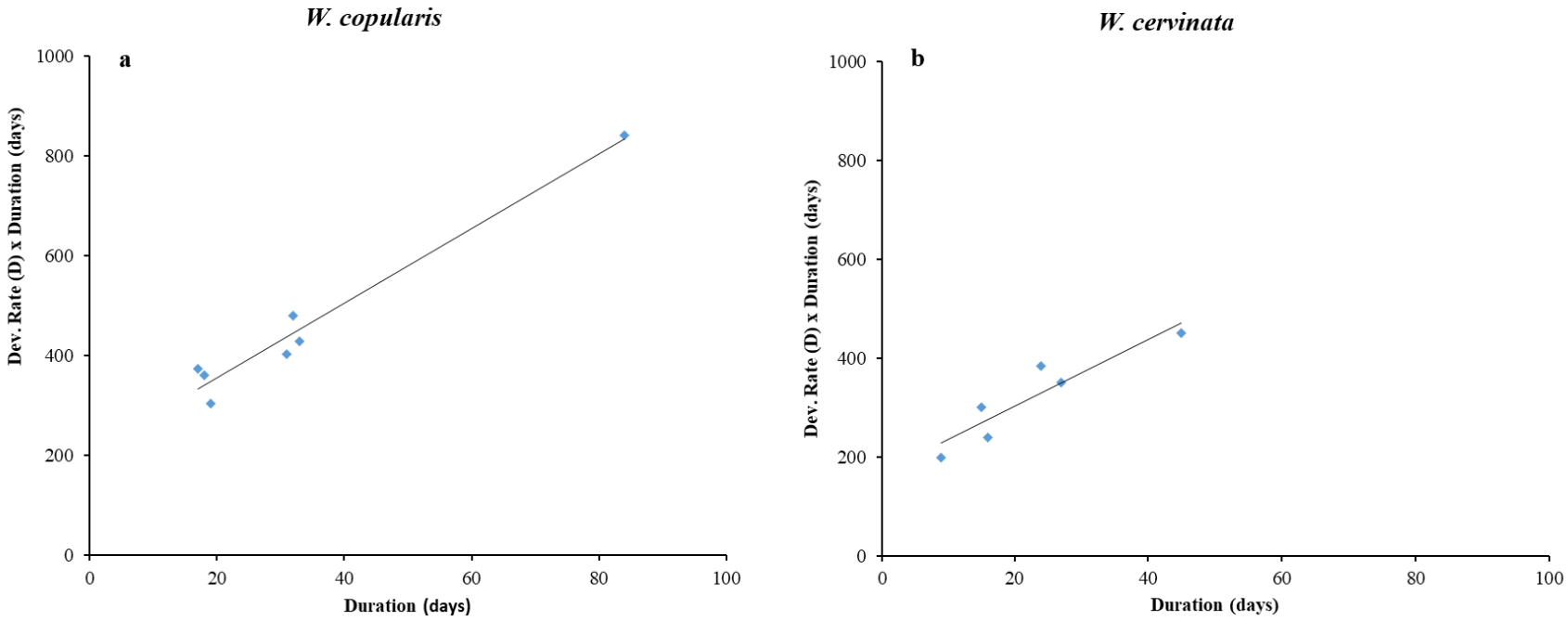


Figure 9.3: The Ikemoto-Takai linear regression model for a) *W*. *copularis* and, b) *W*. *cervinata*.

9.4.2 Nonlinear models

Fit statistics and parameter estimates for the Briere-1 and Lactin models are shown in Table 9.3, and the fitted models for both species are shown in Figure 9.4.

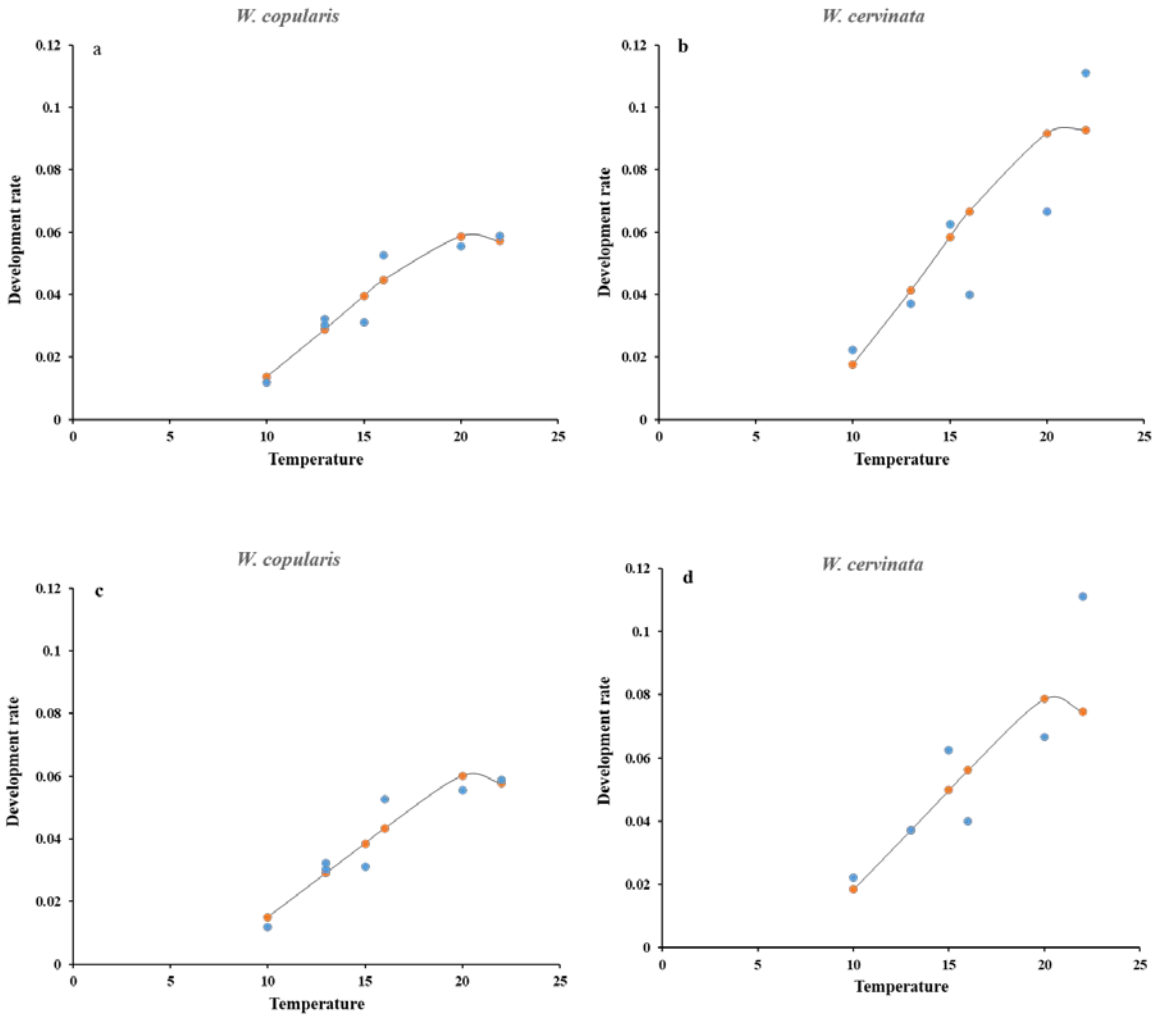


Figure 9.4: Briere-1 model fitted to data for a) *W. copularis* and b) *W. cervinata* and the Lactin model for c) *W. copularis* and d) *W. cervinata*.

For *W. copularis* both nonlinear models fitted the data well with an R^2 of 0.97 for Briere-1 and R^2_{adj} of 0.95 similar to the Lactin model with R^2 = 0.96 and R^2_{adj} 0.93. The AIC and RMSE were comparable for both models where AIC and RMSE were -68.72 and 0.0057 respectively for Briere-1 and slightly higher at -65.82 and 0.0068 respectively for the Lactin equation. For *W. cervinata* the Briere-1 model provided a slightly better fit of the two non-linear models with R^2 = 0.88 and R^2_{adj} 0.82 compared with R^2 = 0.83 and R^2_{adj} = 0.81 for the Lactin equation. Additionally, the AIC and RMSE for the Briere-1 model were the lowest at -42.93 and 0.0021 respectively, compared with -40.34 and 0.0025 for the Lactin equation. Interestingly when the Briere-1 is compared with the Ikemoto-Takai model, they both provided a similar fit to the data.

The results for the developmental parameter estimates provided by the models are shown in Table 9.4. Of particular interest are the lower threshold for development (T_0) and the thermal constant (DD) highlighted in Table 9.4. All models provided similar estimates for the three cardinal temperatures (the lower threshold for development, the optimum and maximum temperatures) within and between species, when they could be calculated, thus giving high confidence in the results. However, although the estimated thermal constants where higher for *W. copularis* than *W. cervinata* regardless of the model used, the estimates between models also differed within species (Table 9.4).

Table 9.4: Parameter estimates for all models. Numbers in brackets are the standard errors for the developmental parameters for the linear models.

Models	**Parameter**	***W. copularis***	***W. cervinata***
Linear	*a*	-0.028	-0.046
	b	0.0043	0.0063
	T_0	6.4 (1.79)	7.1 (2.29)
	DD	228 (47.9)	156 (38.0)
Ikemoto-Takai	T_0	7.5 (0.65)	6.8 (1.43)
	DD	205 (26.02)	167 (36.3)
Briere -1	a	0.000096	0.00015
	T_0	6.3	7.0
	T_{max}	25.0	25.5
	T_{opt}	20.7	21.2
Lactin	p	0.0045	0.0058
	T_{max}	26.9	26.6
	ΔT	1.1395	1.134
	λ	-1.03059	-1.042
	T_0	6.75	6.9
	T_{opt}	20.9	20.8

9.5 Discussion

Temperature as a critical factor influencing the rate of development of poikilothermic organisms including insects is well known (Welch 1978). Because of this relationship, temperature has a profound effect on the population biology, distribution and abundance of all insect species. This research was aimed at estimating important developmental parameters, the lower threshold for development (T_0) and the thermal constant (DD) for egg development for *W. copularis* and *W. cervinata* given their status as important pasture pests. The ultimate objective is to develop a simple phenology model that

could improve the timing of application of control measures to minimise economic damage to pasture. In particular for more accurate timing of the application of the IGR, diflubezuron (Ferguson 2000). The original objective was to study not only egg hatch but the development of all the larval stages for both species. However, it became apparent that the time and technical resources required to complete such a study was beyond the scope of this research. This was limited by the considerable resources required to keep an appropriate sample size for each species alive throughout their long larval development, as well as monitor the development of each individual insect with enough precision (daily) to develop a robust model.

Few thermal development studies have been carried out on *Wiseana sp.* and even fewer on *Wiseana* species that have been accurately identified. Two studies that did attempt to identify the *Wiseana* species involved was firstly that by Dumbleton (1945) who studied aspects of the life history and habits of what he called *Wiseana cervinata.* However, that research does not explain how the species were actually identified and in view of Dugdale's 1994 revision of the genus his identification has to be tentatively accepted at best. The second, more extensive and detailed study was carried out by Ferguson & Crook (2004) who examined the development of both *W. copularis* and *W. cervinata* (identified by examination of the bursa copulatrix as per Dugdale 1994) from egg to adult. The time taken for egg hatch at four constant temperatures was studied in detail with records made daily and larval instar development monitored weekly. Ferguson & Crook (2004) did not fit a model to determine a lower threshold for development (T_0) or thermal constant (DD) for either species, but did suggest the development rates for each species was similar. Dumbleton (1945), however, did estimate a lower threshold for development for *W. cervinata* (44°F or ~ 6.7°C) but, while in agreement with the values calculated in this study, did not describe how this parameter was calculated.

The IGR diflubenzuron is currently the most common control method for porina (Ferguson & Crook 2004). The timing of application of this pesticide is critical for it to be effective. The mode of action of diflubenzuron is by interfering with chitin deposition, and it needs to be ingested shortly before the larvae moult. The efficacy of diflubenzuron is greatly enhanced if it is applied when the larvae are small and frequently moulting (Ferguson & Crook 2004). This chemical also degrades quickly after application, as it has a half-life of less than seven days, estimated by Tomlin (1994). French & Pearson (1979) tried to develop models to determine the dates of peak egg hatch under field conditions using light trap data and climatological data, but unfortunately the species they studied were not identified and may have consisted of eggs from multiple species. Their approach was to use peak flight as a biofix for peak egg laying on the soil surface, then egg development times in relation to temperature to predict peak egg hatch in the field. The model used by French & Pearson could be regarded as non-standard, but their methods 3 and 4, in theory, were similar to a degree-day approach for timing development.

In this study, two linear models (the standard model and the Ikemoto-Takai model) were compared for goodness of fit to the developmental data for *W*. *copularis* and *W*. *cervinata*. Other important parameters such as the optimum temperature for development (T_{opt}) and the lethal upper temperature (T_{max}) were estimated by the two non-linear models Briere-1 and the Lactin equation. Such parameters along with T_0 indicate the range of temperatures over which the species might survive and may be useful for studies interested in predicting insect distribution and response to changing climate. Given the contrasting temperatures larvae experience over their approximately nine months of development (high while larvae are surface dwelling in summer and low when larger larvae have formed deep burrows in winter), T_{opt} may change for different development stages, but defining that is beyond the scope of this study. The main purpose for fitting the two nonlinear models was to help verify an appropriate value for the most important developmental parameter, T_0. The Ikemoto-Takai model gave the highest estimate of T_0 for *W. copularis* at 7.5℃ compared with 6.4℃ for the standard model. The Briere-1 and Lactin equation gave estimates of T_0 as 6.3℃ and 6.7℃, respectively. For *W cervinata* the estimates were very similar ranging from 6.8℃ (Ikemoto-Takai model), to 6.9℃ (Lactin equation), 7.0℃ Briere-1 to 7.1℃ (standard linear model). Overall the nonlinear models help verify an appropriate value for T_0. The approximate 95% confidence intervals for T_0 for *W. copularis* is (3.5℃, 9.9℃) for the standard linear model and encloses the 95% confidence interval for the estimated T_0 from the Ikemoto-Takai model (5.8℃, 9.2℃). Similarly, there is considerable overlap between the 95% confidence intervals of both linear models for *W*. *cervinata*. For the standard model it was (4.5℃, 11.6℃) and (3.9℃, 9.6℃) for the Ikemoto-Takai model.

The estimated lower thresholds for larval development ranged from 6.3 to 7.5℃ depending on species and model used. Porina larvae in Canterbury only experience such temperatures in mid-winter (Macara 2016) at which time the larvae are living in well-established burrows up to 30 cm or deeper in the soil. The models suggest, therefore, that development may slow and even stall during winter. T_{max} for both species was estimated to be between 25℃ and 26.9℃, although air temperatures in Canterbury regularly exceed these (e.g. Macara 2016). Porina larvae will be at most risk to these in summer and early autumn while they are small and dwelling on or near the soil surface. However, it is probable that the effects of such temperatures are mediated by pasture cover and larval behaviour especially when their burrows have penetrated the soil where air temperatures are buffered by the soil mass. Soil temperatures in lowland Canterbury, at 10 cm depth, do not reach the estimated T_{max}. This implies that once larval burrows are in the soil profile, the larvae are buffered from lethal high temperatures.

Of the two linear models, the Ikemoto-Takai model had a better fit and lowest standard error for the parameters of interest. Thus, the Ikemoto-Takai model was chosen as the best model to provide critical parameters T_0 and DD for egg development of *W. copularis* (7.5℃, 205 DD) and *W. cervinata* (6.8℃, 167 DD) to design a phenology model (Chapter 10) to improve their control in pastures in New Zealand.

Chapter 10

A phenology model for predicting the optimal time for applying control measures for the pasture pest, *Wiseana* (Lepidoptera: Hepialidae) in some parts of New Zealand

10.1 Abstract

Since the identification of *Wiseana* species as pests, attempts have been made to improve predictions of the species phenology to increase control efficacy. This has largely relied on field observations and conditions related to general population development to predict lifecycle events that can improve timing of control measures. This approach, however, suffers from lack of actual developmental data. Here, development data for both eggs and larvae of the two dominant pest species (*W. copularis* and *W cervinata*), reared under constant temperatures, were used to generate a prototype forecasting model to be able to estimate the optimal control period for each species based on physiological time in the form of degree days (DD). The lower threshold for development (T_0) and thermal constant or sum of effective temperatures (SET) in combination with historical light trap and weather data were used to develop a timing model for peak flight at different localities in New Zealand. The predicted peak flight at ten sites in New Zealand was then used to forecast the optimal time at those sites to apply control to *W. copularis* and *W. cervinata*. The results were validated by comparing predictions with observed peak flights using leave-one-out cross-validation. The results from this study are expected to set the foundation for future research to improve control of damaging populations of porina in pastoral farming in New Zealand.

Key words: Phenology, degree days, lower threshold, weather data, peak flight, prediction

10.2 Introduction

Ever since *Wiseana* species were identified as pests, attempts have been made to improve predictions of the species phenology to increase control efficacy. A few studies attempted to relate field observations and conditions to population development (Dumbleton 1945, Helson 1967) while others recorded *Wiseana* sp. development under constant temperature conditions (Dumbleton 1945, French & Pearson 1979, Ferguson & Crook 2004). The latter studies tried to use development rate models to improve timing of insecticide control and, in more recent decades, application of less environmentally harmful methods (Stewart & Ferguson 1992, Ferguson 2000). All studies prior to 1994 and Dugdale's

revision of the genus suffered from lack of knowledge of exactly which species had been collected and/or studied, such that robust recommendations were lacking. Ferguson & Crook (2004) were the first to try to determine developmental information for correctly identified species, namely, *W. copularis* and *W. cervinata*, where they studied the development times of the egg and larval stages of these species under constant temperatures in the laboratory. Despite these investigations, the detailed timing of insecticide applications have been based partly on field observations as well as a "best guess" arising from the Ferguson & Crook (2004) research. Ferguson & Crook (2004) suggested monitoring the flight period of *Wiseana* spp. ideally coupled with field sampling, to determine the optimal period of application of the insecticide diflubenzuron. As an insect growth regulator (IGR), this control measure targets the vulnerable early instar larvae and if no sampling was conducted, to apply within three months of estimated egg hatch. To date, only one attempt was made by French & Pearson (1979) to use the physiological time that relates moth development directly to temperature for forecasting and predicting peak moth flight and, therefore, peak egg hatch. Using peak moth flight gives a bio-fix (Worner 1998, Welch *et al*. 1978) for predicting peak egg hatch and therefore larval emergence with the aim of more accurately timed application of pest control.

The study by French & Pearson (1979) provided four methods to time larval emergence of porina in the field based on their study of egg development in relation to temperature. It is unfortunate that not only were the actual species studied not identified, but also the way their development versus temperature relationships were developed and utilised was likely to introduce additional levels of prediction error on top of sampling or experimental error. Despite these flaws, at the time their study was an advance on previous research. Specifically, in their "Method 1", French & Pearson (1979) fitted a linear regression equation to the time versus temperature relationship for egg hatch under constant temperatures in the laboratory, which was clearly curvilinear. To predict the incubation period of porina eggs in the field, the mean temperature for a particular month was inserted into the linear regression equation derived to estimate the period of incubation. Method 2 involved hand drawing the curve to read off the incubation period using the mean monthly temperature in the field for the period of interest. The third and fourth methods were based on calculating proportional development per day in relation to temperature, much like the physiological time or degree-day concept (Chapter 9). However, French & Pearson (1979) used the straight line fitted to the development time versus temperature, a nonlinear relationship (Method 3), or the hand-drawn curve (Method 4) and a lower threshold (T_0 = 10°C) attributed to Dumbleton (1945), which appears incorrect in their calculations. In all four methods, peak egg hatch was estimated by adding the calculated incubation period to the date of peak moth flight (French & Pearson 1979).

A more detailed study of development of two major pest species*, W. copularis* and *W. cervinata,* was carried out by Ferguson & Crook (2004). Both egg hatch and larval development were studied in detail

under constant temperature conditions. Results of the egg hatch data there were used to generate the important developmental parameters reported here in Chapter 9. The actual data from experiments carried out by Ferguson & Crook (2004), who recorded the development of the larvae of the two species in relation to constant temperatures, are used in this study to develop a prototype forecasting model to estimate the optimal control period for each species based on physiological time. Physiological time is used because forecasting population events, such as peak flight or peak hatch, based on calendar days often gives estimates with very wide confidence intervals which thus lack precision. Clearly, weather conditions, especially temperature fluctuations over time, will be key factors that will determine the actual date (French & Pearson 1979, Worner 1992). Physiological time, in particular the degree-day approach based on temperature dependent developmental data, is the simplest way to account for the influence of temperature on the rate of growth and development of insect populations. This means phenological events or species phenology can be forecast in different localities where seasonal temperature conditions vary. In this chapter, physiological time in the form of degree days (DD) is used to investigate its utility to refine predictions of when the vulnerable larval stages of the two *Wiseana* species are likely to be present in the field so that control can be more efficiently targeted.

The first objective in this research was to use the developmental lower threshold for development (T_0) and thermal constant or sum of effective temperatures (SET) determined in Chapter 9, in combination with weather data, to predict the optimal time to apply diflubenzuron to *W. copularis* and *W. cervinata* for ten sites in New Zealand. This IGR must be applied so that it is consumed close to moulting as it has a short half-life of seven days. Since moults are more frequent during early instar development (Ferguson & Crook 2004), it is important to estimate when these are likely to occur, so an optimal application time can be determined. This can be forecast from the time of peak flight, however, flights need to be finished to estimate what the time was of the peak flight, but actual flights may not have finished in time to be able to calculate this. Therefore, the utility of using historical data of light trap records along with weather data was investigated to develop a timing model for predicting peak flight for ten different localities in New Zealand. The results of both these objectives are expected to set the foundation for future research to improve control of damaging populations of porina in pastoral farming in New Zealand.

10.3 Method

10.3.1 Developmental data

The results of the egg development studies are described in Chapter 9. In that study, important developmental parameters for egg hatch were estimated. These were T_0 and SET for egg hatch of 7.5℃ and 205 DD respectively for *W. copularis*, and 6.8℃ and 167 DD respectively for *W. cervinata*. To

determine the optimum time for control, this data was combined with larval development information recorded by Ferguson & Crook (2004). In that study, approximately 100 eggs were placed in constant temperature conditions (10, 13, 16 and 20℃) and monitored until they died or pupated while developing through a number of larval instars. Determination of the instar number was by head capsule width and each individual was measured every 20 days.

In some cases, the monitoring was done more frequently (every 10 days) despite the serious constraints of handling small larvae. Porina are considered most vulnerable from the 1st to 4th larval instar when they are close to the soil surface. From the 5th larval instar, the time between moults is too far apart for the IGR to be reliably effective, taking into consideration the half-life of the IGR, and the level of control obtained consequently decreases (Colin Ferguson, personal communication, AgResearch, NZ). The data were therefore used to determine degree day requirements for larval development up to both the mean 4th and 5th instars to give maximum information about the vulnerable period.

10.3.2 Determining a lower threshold for development of larvae

The data compiled by Ferguson & Crook (2004) were used to estimate the average DD required for development to the 4th and 5th instars for both species. While Ferguson & Crook (2004) recorded the larval development of each species over a range of temperatures, observations of early instar larval development were mostly carried out every 20 days. Unfortunately, this does not allow the determination of a robust temperature-dependent development relationship to calculate important developmental parameters. Daily observations are usually required to achieve this. Thus, the precision of the larval development data did not allow calculation of a sensible T_0. This is not an unusual problem. The application of the thermal requirements in phenology and climate models is often limited by data availability. However, despite the lack of data, decisions still need to be made, especially in the areas of pest management and pest risk assessment where some attempt is needed to predict the timing of insect life cycle events (Worner & Gevrey 2006, Worner *et al*. 2015). In such cases, Preuss (1983) was possibly the first to advocate using standardized thresholds for insect development, but not necessarily in the absence of appropriate data. Preuss (1983) argues strongly for a compromise between utility and the quest for precision, which may be unattainable anyway. Preuss (1983) suggests using standard thresholds at 5, 10 and 15℃ that would suffice for many agronomic applications claiming that degree day methods, even with different thresholds, are closely correlated. Thus, standardisation would lead to greater utility of the models especially when used to examine relationships between insects and plants (Preuss 1983). A publication by Jarošík *et al*. (2002) challenged the widely held understanding that, within a species, each developmental stage has its own, specific lower developmental threshold. Jarošík *et al*. (2002) in a meta-analysis of 426 populations of 349 species, found that the proportion of

the developmental time spent in individual developmental stages of a species does not change with temperature for 57% of the 426 populations. That can only happen if T_0 remains the same for all developmental stages. This concept is called developmental rate isomorphy and has been observed in other ectotherms (Boukal *et al*. 2015). However, Boukal *et al*. (2015) express doubts about the universal validity of the concept, highlighting seasonal constraints on development and variation in photoperiod can (among other influences) lead to developmental rate isomorphy violation. Another approach to lack of data is to use the T_0 for a closely related species as the thermal requirements are similar for related taxa (Jarošík *et al*. 2002, Ikemoto 2003, Dixon *et al*. 2005, Kiritani 2006). Unfortunately, a literature search failed to find any useful information for any hepialid species about development in relation to temperature. However, Nietschke *et al*. (2007) found that for 109 Lepidoptera species whose data had been collected for a database to support phenology models, the mean temperature was 9.6℃ with a range between 3 and 17.5℃. Also, many researchers recognize that there are errors in estimating T_0, despite robust experimentation. For example, Campbell *et al*. (1974) points to the low precision of estimates of lower temperature thresholds which often have a high relative standard error. In this study, the standard errors calculated for the two species in Chapter 9, Table 10.4, resulted in quite wide 95% confidence intervals (6.2 to 8.8℃) for *W. copularis* and (4.0 to 9.6℃) for *W. cervinata*. A practical approach of using a nominal threshold if one is not exactly known has been proposed also by Jarosik *et al*. (2002) in the area of pest risk analysis. Jarosik *et al*. (2002) reported the average value of the lower developmental threshold for N = 1,555 populations of 961 species in a large thermal database was 10.19℃, with standard deviation = 3.84℃. In a later study, using a database of 1,054 species, Jarosik *et al*. (2011) found that the mean lower developmental threshold for N = 2,393 populations of 968 species was 10.2℃, with a range of 0-28.1℃. As with the former database, the larger database comprised developmental times at various constant temperatures for insect and mite species from all over the world.

Despite the apparent support for using a T_0 of ~10℃ it was decided to use the egg development thresholds determined in Chapter 9 as nominal lower thresholds for the early larval instars for each species. This decision was based on the knowledge that *Wiseana* are alpine cold adapted species (Brown *et al.* 1999) and are unusually subterranean for much of their life cycle. They have evolved in an environment where temperatures are lower than air temperatures to which most other lepidoptera are exposed, therefore a T_0 of 10℃ is likely too high. Furthermore, there is more recent evidence for the existence of developmental isomorphy (e.g. Honek *et al*. 2003, Zahiri *et al.* 2010, Kuang *et al*. 2012). While there are reports of violations of developmental isomorphy for individual species, as well as continued debate (Kutcherov *et al.* 2011, Boukal *et al*. 2015), Shi *et al*. (2010) report developmental isomorphy violation when appropriate statistics are used. Using what they claim as appropriate statistics, Shi *et al*. (2010) report the existence of developmental isomorphy from developmental rate

data of *Lygocoris lucorum* Meyer-Dur (Hemiptera: Miridae) for its egg and nymphal stages which were shown to differ as much as 9.27℃ ± 0.34 and 6.78℃ ± 1.02 respectively. Jarosek *et al*. (2002) also claim that often when developmental isomorphy violations are found they are extremely small or can be challenged. The discussion gets more bewildering even when developmental rate isomorphy is supposedly carefully tested and found not to be present, which on closer examination is shown to be wrong. For example, Kutcherov & Kipyatkov (2011) in a very detailed study of the developmental rates of the dock leaf beetle *Gastrophysa viridula* (De Geer) (Coleoptera: Chrysomelidae) to carefully test for developmental isomorphy, found violation of developmental isomorphy, based on significant differences in T_0 between the egg, larval and pupal stages. The authors analysed reported mean development times for eggs, larvae and pupae at different temperatures (see data Kutcherov & Kipyatkov, 2011, Table 2). For each stage there were non-significant differences between the proportions of total development time at each temperature within the stage, thus supporting the presence of developmental isomorphy. Indeed, emphasis on the precision of many developmental studies may well be unwarranted as there is known bias estimating T_0 and SET caused by imprecise measurement of development at extreme temperatures (van Rijn *et al.* 1995, Zahiri *et al*. 2010). Additionally, while some may find significant differences between developmental parameters, such as in the Kutcherov & Kipyatkov (2011) study, such differences may be well within the normal errors encountered in a practical phenological setting. Such settings often involve significant experimental, measurement and sampling errors, Additionally, Rebaudo & Rabhi (2018) in their recent review of modelling temperature-dependent development rate and phenology in insects, suggest the problem of precision is further compounded by the disregard for the dependence of development rate on multiple influencing factors.

10.3.3 Determining the degree day requirements for development to 4th and 5th instars

Ferguson and Crook (2004) studied the development of 100 individuals from egg hatch over time at each temperature (10, 13, 16 and 20℃). The day on which individuals in the sample were at the mean 4th and 5th instars at each temperature was recorded. Because temperatures were constant, it was possible to calculate the approximate number of degree days required for development for the average individual in both instars. The number of days from egg hatch at each temperature to mean 4th and 5th instar development was converted to DD by the formula [DD = number of days x (Temperature - T_0)] where T_0 = 7.5℃ for *W. copularis* and 6.8℃ for *W. cervinata*. Because of variation between the developmental responses to each temperature, the DD required for larval development at each temperature was averaged over the temperatures.

10.3.4 Predicting the period for optimal control in the field using IGR

Peak flight, here, was defined as the day on which 50% of the cumulative seasonal flight had occurred, which was determined retrospectively from actual flight data. From data developed in Chapter 3 and a number of site-years data in New Zealand (C. Ferguson unpublished), where the numeric peak (i.e. the night on which the greatest number of moths flew) occurred later in the season when a considerable number of individuals had been caught already, and presumably the females had laid a large number of eggs. For Lincoln 2016/2017, Waimahaka S 1998/1999 and Manawatu 2012/2013, 80%, 76% and 74% of moths respectively had flown before the numerical peak was recorded. For all other sites, peak flight based on 50% trap catch was equivalent or very close to the recorded numerical peak. Using peak flight as a proxy for peak egg laying, DDs or SET from peak flight to egg hatch, using the SET were determined in Chapter 9, and DDs from egg hatch (peak) to mean 4th and 5th instars were used to calculate the period over which the larvae are likely most vulnerable to IGR control. The optimal period was determined as starting on the day that peak egg hatch occurs until the dates on which the mean 4th or 5th instar, whichever stage is the most informative, occurs. The optimal periods for both study species over a range of sites in New Zealand, for which data were available, were calculated using local weather data obtained from NIWA and records of observed porina flights over one or two seasons, depending on the site (Table 10.1). Unfortunately, there is no easy way of observing and recording the development of porina eggs or larvae while on the surface of the soil because they are so small, which meant there were no data for validation of the model. The intention here is to determine if the current work could provide useful information for future study. Flight data for the two Lincoln sites were collected as part of this research. The flight data for all other NZ sites was provided by Colin Ferguson, Invermay Agricultural Centre, Mosgiel, New Zealand.

Using peak flight as a proxy for peak egg laying, the DD or SET for egg hatch (Chapter 9) were added to peak flight to estimate peak egg hatch (= larval emergence). Degree days, according to the T_0 for each species were accumulated from the date of peak flight until the total degree days required for egg hatch for each species occurred. Required DD or SET for egg hatch was 205 DD using T_0 = 7.5℃ for *W. copularis,* and 167 DD for *W cervinata* with T_0 = 6.8℃ (Chapter 9). Peak egg hatch marked the beginning of the period of optimal control. Degree days from egg hatch or larval emergence to mean 4th instar and 5th instar were added to peak egg hatch to calculate the end of the period over which the larvae are most vulnerable. Only four sites had sufficient individuals of *W. cervinata* caught in traps that allowed a workable analysis for them.

10.3.5 Temperature data

Air and soil temperature data were obtained from weather station summaries published by National Institute of Water and Atmospheric Research of New Zealand (NIWA, https://cliflo.niwa.co.nz) (Table

10.1). Because young larvae are surface or close to surface dwelling, air temperatures were used to determine the period of optimal control using IGR. To predict peak flight as moths emerge from pasture, both daily soil temperatures and daily mean air temperatures for each site were sourced from weather stations nearest each field. The mean of each temperature variable was used in the daily degree-day (Celsius) calculation for (DD = Mean T - T_0 x 1 day).

Table 10.1: Summary of sample sites for weather and porina light trapping data at various New Zealand locations used in this study.

Years	Locations	Weather station
1997-98	Waimahaka S	Tiwai Point Ews
1997-98	Waimahaka P	Tiwai Point Ews
1998-99	Waimahaka S	Tiwai Point Ews
1997-98	Mahinerangi	Dunedin Aero Aws
1998-99	Mahinerangi	Dunedin Aero Aws
1998-99	Lumsden	Lumsden Aws
1999-2000	Lumsden	Lumsden Aws
2012-2013	Manawatu	Flat Hills Wxt Aws
2015-2016	Lincoln	Lincoln, Broadfield Ews
2016-2017	Lincoln	Lincoln, Broadfield Ews

Degree days, according to the lower threshold for each species, were accumulated to peak flight date (defined above) at each of the 10 sites from a mid-winter bio-fix (July 1). A mid-winter bio-fix was used based on the assumption that little development of the subterranean larvae occurs at that time because temperatures both in the soil and above ground are often below the lower development threshold. Accumulation of DD from mid-winter starts to pick up as mean temperature rises into spring, increasing potential for larval development as spring progresses. DD accumulations for soil temperatures recorded at 10 cm depth and for air temperature were compared. Despite time lags and differing amplitudes in the diurnal pattern between the two environments, there is often a strong correlation between air temperature and soil temperature (Zheng *et al*. 1993). Also, air temperatures are often more convenient for practical application when there are no soil temperature data available for nearby sites. Furthermore, soil temperature is a variable that links surface structure to soil processes and is subject to interaction of a range of meteorological conditions and physical characteristics creating poorly understood heterogeneity (Kang *et al*. 2000). Using T_0 = 7.5℃ for *W. copularis* and 6.8℃ for *W cervinata*, total degree days between mid-winter and peak flight for each species, site and season (Table 10.1), and the Julian date (days of the year) of peak flight were recorded for each temperature variable (soil and air). The average calculated Julian calendar date and average

degree days between mid-winter and peak flight were used to predict peak flight for each species-site-year combination in a leave-one-out validation.

Leave-one-out cross-validation (Cawley & Talbot 2004, Lankin-Vega *et al*. 2008) allows the generation of a validation dataset that is independent of the data used to develop the DD models. For leave-one-out validation, nine site-years were combined to calculate the average Julian date and average degree days of peak flight. The remaining 10th (left-out) site-year was used to test the prediction given by the model for validation. This process was repeated for all the site-year combinations. The result was 10 different data sets to provide 10 models and 10 site-years for model validation. Three are given as follows:

1) Combined nine site-years (Waimahaka S (1997-98), Waimahaka P 1997-98, Waimahaka S (1998-99), Mahinerangi (1997-98), Mahinerangi (1998-99), Lumsden (1998-1999), Lumsden (1999-2000), Manawatu (2012-2013), Lincoln (2015-2016)) to validate for Lincoln (2016-2017).

2) Combined nine site-years (Waimahaka S (1997-98), Waimahaka P 1997-98, Waimahaka S (1998-99), Mahinerangi (1997-98), Mahinerangi (1998-99), Lumsden (1998-1999), Lumsden (1999-2000), Manawatu (2012-2013), Lincoln (2016-2017) to validate for Lincoln (2015-2016).

3) Combined nine site-years (Waimahaka P 1997-98, Waimahaka S (1998-99), Mahinerangi (1997-98), Mahinerangi (1998-99), Lumsden (1998-1999), Lumsden (1999-2000), Manawatu (2012-2013), Lincoln (2015-2016), Lincoln (2016-2017 to validate for Waimahaka S (1997-98).

10.4 Results

10.4.1 Optimal period for control

The total degree days required for egg hatch for each species (Chapter 9) with T_0 = 7.5℃ for *W. copularis* and with T_0 = 6.8℃ for *W*. *cervinata* are shown in Table 10.2. Degree days from egg hatch to mean 4th and 5th instars for each species are also shown and the calculated DD from peak flight to mean 4th and 5th instars are given (Table 10.2).

Table 10.2: Degree days after peak flight to when the porina larvae are most vulnerable to IGR application.

***Wiseana* species**	**DD from peak flight to peak egg hatch**	**DD from peak egg hatch to mean 4th instar**	**DD from peak egg hatch to mean 5th instar**	**DD from peak flight to mean 4th instar**	**DD from peak flight to mean 5th instar**
	(1)	**(2)**	**(3)**	**(1)+(2)**	**(1)+(3)**
W. copularis	205	362	389	567	594
W. cervinata	167	381	543	548	710

These DD totals were then used to forecast the dates for the optimal period for IGR control for *W. copularis* over all ten sites (Table 10.3) and for *W. cervinata* over the four sites that had suitable flight data (Table 10.4).

Table 10.3: Optimum control periods for *W. copularis*

Site	Peak flight	DD from July 1st to peak flight	Optimum control period for *W. copularis*	
			Peak egg hatch to mean 4th instar	Peak egg hatch to mean 5th instar
Waimahaka S 1997/98	18-Jan	494	12-Feb to 9-Apr	12-Feb to 15-Apr
Waimahaka P 1997/98	11-Jan	446	6-Feb to 27-Mar	6-Feb, to 3-Apr
Waimahaka S 1998/99	27-Dec	450	23-Jan to 10-Mar	23-Jan to 14-Mar
Mahinerangi 1997/98	24-Jan	644	11-Feb to 1-Apr	11-Feb to 13 Apr
Mahinerangi 1998/99	13-Jan	715	8-Feb to 19-Mar	8-Feb to 22-Mar
Lumsden 1998/99	23-Jan	701	17-Feb to 17-May	17-Feb to 3-Jun*
Lumsden 1999/2000	8-Jan	502	9-Feb to 26-Apr	9-Feb to 3-May
Manawatu 2012/2013	8-Jan	605	8-Jan to 13-Feb	8-Jan to 16-Feb
Lincoln 2015/2016	16-Jan	819	6-Feb to 11-Mar	6-Feb to 15-Mar
Lincoln 2016/2017	19-Dec	632	12-Jan to 19-Feb	12-Jan to 21-Feb

* Not enough degree days accumulate after this date and before end of June to reach the total DD required.

Table 10.4: Optimum control periods for *W. cervinata*

Site	Peak flight	DD from July 1st to peak flight	Optimum control period for *W. cervinata*	
			Peak egg hatch to peak 4th instar	Peak egg hatch to peak 5th instar
Mahinerangi 1997/98	21-Nov	283	18-Dec to 1-Feb	18-Dec to 15-Feb
Mahinerangi 1998/99	22-Nov	392	19-Dec to 27-Jan	19-Dec to 5-Feb
Lumsden 1998/99	23-Jan	803	13-Feb to 29-Mar	13- Feb to 30 Mar*
Lincoln 2016/2017	31-Oct	345	24-Nov, 7-Jan	24-Nov to 22-Jan

* Not enough degree days accumulate after this date and before end of June to reach the total DD required.

Additionally, average dates for the optimum period for each species were calculated, using Julian dates. Because the flights and early development of porina span the New Zealand summer period from one year to the next, the Julian dates were greater than 365 in some cases. The average period for optimal control over all site-years for *W. copularis* was calculated to extend from 2nd Feb to 25th Mar for peak egg hatch to mean 4th instar and 2nd Feb to 1st Apr for 5th instar. The average period for *W. cervinata* corresponds to its earlier development where the period from peak hatch to mean 4th instar is 27th Dec to 8th February and from 27th December to 18th February for 5th instar. Figure 10.1 shows the overlap between these periods for the two species plotted against Julian date. Taking both species together the period from earliest peak egg hatch (*W. cervinata*) to latest 5th instar (*W. copularis*) extends from 27th Dec to 1st April, slightly more than three months. For *W. copularis* the average number of days from peak hatch to mean 4th and 5th instar was 51 ± 5.8 days and 58 ± 7.0 days, respectively. For *W. cervinata* the average period in days from peak hatch to mean 4th and 5th instars was 43 ± 1.4 and 53 ± 3.5 days, respectively.

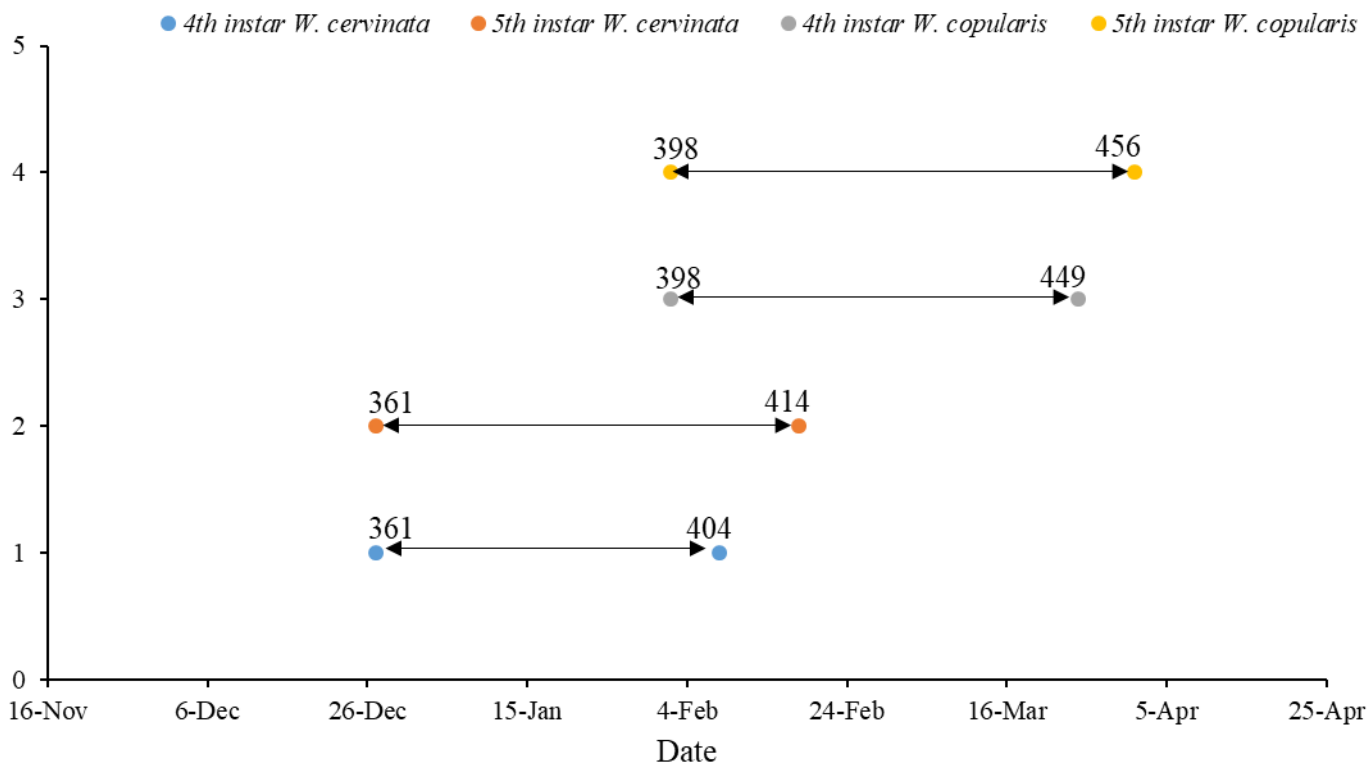


Figure 10.1: The average optimal control period from peak egg hatch to peak 4th and 5th larval instar for *W. cervinata* and *W. copularis*. Points are labelled using Julian dates and actual date labels are shown on the x-axis.

10.4.2 Predicting peak flight

Only four sites had suitable data for calculation of *W. cervinata* peak flight, so the analysis was completed only for *W. copularis*. Table 10.5 shows the observed and leave-one-out predicted dates of peak flight for both the mean Julian date models (averaged over nine sites) and mean degree day models (averaged over nine sites) for all 10 site-years using soil temperatures. Table 10.6 shows the same results for air temperatures. The absolute mean (ABS) [abs(observed - predicted)] error is perhaps the most meaningful measure of accuracy, where it gives the average error of prediction in days to be expected over all site-years. For soil temperatures, the absolute mean error of prediction was 12 ± 2.4 days for the Julian date method and 16 ± 5.2 days for the degree day method. The range of prediction errors for the Julian date model varied from 0 to 22 days and for the DD method from 0 to 53 days, with the largest deviation for Lincoln 2015/16 season (Table 10.5). For air temperatures, the absolute mean error of prediction was 12 ± 2.4 days for the Julian date method and 14 ± 2.6 days for the degree day method. The range of prediction errors for the Julian date model varied from 0 to 22 days and for the DD method from 1 to 27 days again, with the largest deviation for Lincoln 2015/16 season (Table 10.6).

Table 10.5: Leave-one out predictions for each site being validated using soil temperatures (10 cm depth). A prediction for each site is made based on the average Julian date or DD calculated on the remaining site data. Absolute mean errors (ABSME) are given in days.

Sites	Observed Peak flight	Predicted using mean Julian date	Predicted using mean DD	ABS (observed-predicted) Julian date	ABS (Observed-predicted) DD
Waimahaka S 1997/98	18-Jan	7-Jan	9-Feb	11	22
Waimahaka P 1997/98	11-Jan	8-Jan	9-Feb	3	29
Waimahaka S 1998/99	27-Dec	10-Jan	17-Jan	14	21
Mahinerangi 1997/98	24-Jan	6-Jan	9-Feb	18	16
Mahinerangi 1998/99	13-Jan	8-Jan	1-Jan	5	12
Lumsden 1998/99	23-Jan	7-Jan	23-Jan	16	0
Lumsden 1999/2000	8-Jan	8-Jan	10-Jan	0	2
Manawatu 2012/2013	20-Jan	10-Jan	24-Dec	21	4
Lincoln 2015/2016	16-Jan	7-Jan	24-Nov	9	53
Lincoln 2016/2017	19-Dec	10-Jan	18-Dec	22	1
				ABSME 12	ABSME 16

Table 10.6: Leave-one out predictions for each site being validated using air temperatures. A prediction for each site is made based on the average Julian date or DD calculated on the remaining site data. Absolute mean errors (ABSME) are given in days**.**

Sites	Observed Peak flight	Predicted using mean Julian date	Predicted using mean DD	ABS (observed-predicted) Julian date	ABS (Observed-predicted) DD
Waimahaka S 1997/98	18-Jan	7-Jan	2-Feb	11	15
Waimahaka P 1997/98	11-Jan	8-Jan	2-Feb	3	22
Waimahaka S 1998/99	27-Dec	10-Jan	17-Jan	14	21
Mahinerangi 1997/98	24-Jan	6-Jan	17-Feb	18	7
Mahinerangi 1998/99	13-Jan	8-Jan	31-Dec	5	13
Lumsden 1998/99	23-Jan	7-Jan	10-Jan	16	13
Lumsden 1999/2000	8-Jan	8-Jan	23-Jan	0	15
Manawatu 2012/2013	20-Jan	10-Jan	19-Dec	21	1
Lincoln 2015/2016	16-Jan	7-Jan	20-Dec	9	27
Lincoln 2016/2017	19-Dec	10-Jan	15-Dec	22	4
				ABSME 12	ABSME 14

10.5 **Discussion**

Ferguson *et al.* (1989) and Ferguson *et al*. (1996) carried out studies to evaluate why diflubenzuron gave inconsistent results in trials in South Otago when it was shown to be effective elsewhere in New Zealand. Ferguson *et al*. (1996) proposed that diflubenzuron efficacy is likely a function of the climatic and environmental factors that could allow one or other porina species to dominate in any one season or location. This also suggested that southern New Zealand, with the greatest diversity of porina species, needed further investigation.

In this chapter, records of porina flights over 10 sites in New Zealand plus the New Zealand National Institute of Water and Atmospheric Research weather data (NIWA, https://cliflo.niwa.co.nz) were linked with *Wiseana* development rate parameters, determined in laboratory studies eslewhere (Chapter 9, Ferguson & Crook 2004). This was used to provide a practical protocol to predict the optimal periods for application of IGR for the main porina pest species at different sites in New Zealand. This optimal period of control depends on knowing when the peak flight period of the porina species took place and hence when peak egg laying occurred and therefore when the vulnerable young larvae are likely to be exposed to the pesticide. To estimate peak flight requires knowing the total number of moths caught up to when the flight period has finished, but the latter is difficult to confirm by observation. Therefore, the potential for predicting peak flight before flight has finished in any one locality and year, rather than relying on empirical data, was investigated here.

The optimal control period can be quite variable from year to year. For example, the earliest start on the 8th Jan occurred in Manawatu 2012/2013 in the North Island, while the latest occurred in Lumsden in the south of the South Island, for the same site for different years, where the optimal control period was later than all other sites. This even extended into May and June for *W. copularis* caused by temperatures dropping below threshold in that region with slow accumulated DD over the later months. However, the average period for each species is likely to be informative. Here it was shown that there was overlap of the optimal periods between species (Figure 10.1), depending on whether mean 4th instar or 5th instar was of interest. Taking both species together the period from the earliest peak egg hatch (4th *W. cervinata*) to the time when instar development slows (5th instar *W. copularis*) extends from 27th Dec to 1st April. The length of the period is similar to the proposed best guess indicated by Ferguson & Crook (2004) who suggested the most vulnerable period can occur over three to four weeks. Ferguson & Crook (2004) did suggest that 5-6 moults would occur over that time and did not comment on timing, recommending instead, that larvae be sampled to determine stage of development and determine densities. Unfortunately, over the vulnerable period, larvae are very small, difficult to find and very delicate to handle for instar assessment, therefore sampling is not easy and has motivated this research. Overlap between the average vulnerable periods for each species

might therefore help inform further optimisation of the application of diflubenzuron by indicating a critical time for maximum efficacy. Clearly, however, using specific information for each individual locality would be ideal to refine predictions further.

Because flights might not finish in time to accurately calculate peak flight, and from there the vulnerable period for control, an objective was to investigate whether degree day calculations could be used to forecast when porina peak flight might occur. On average, for *W. copularis* flights had finished on the 12th Feb for the 10 site-years and 15th Jan for *W. cervinata* at the four useful site-years, overlapping by only a few days in the control periods. On average, *W*. *copularis* had an optimal period for control of 58 days and *W. cervinata* 53 days, giving some time to refine predictions.

In a study carried out on the South Island's West Coast over three summers and 11 sites from 2014 to 2017, Mansfield *et al*. (2017) report that porina moth flights (83% *W. copularis*) typically began in October and continued through into March with peak flight activity from early December to mid-January. This is similar to the observed peak flights for other parts of the South Island and the one North Island site in this research, although here the earliest peak flight was later, on 19th Jan. That could be caused by the differences in how peak flight was defined. In this study, 50% cumulative capture was used because of the likelihood that weather could affect flight activity and the different trajectories of the flight accumulations indicated this definition was more likely to represent the population process better than a numeric peak. On examination of the data, large numbers were often caught late in the season (e.g. Lincoln 2016/2017, Waimahaka s 1998/1999 and Manawatu 2012/2013) such that many moths had already flown and laid eggs before the numeric peak. An appropriate question to ask is whether an activity peak is the same as a population peak? A literature search has so far, for any species failed to show any study that has addressed this question.

The leave one-out validation of the methods used either the mean observed Julian date or the mean observed DDs over nine sites to predict the 10th site left out of the data set. This showed insignificant differences in the precision of the mean predictions for the different methods as well as for DD predictions using different temperatures. T-tests between the two methods (Julian date and DD) for the same temperature variable, were insignificant (soil temperatures*:* $t_{(18)} = 0.72$, $P = 0.48$; air temperatures: $t_{(18)} = 0.53$, $P = 0.59$), as was the t-test between soil temperature predictions and air temperature predictions ($t_{(18)} = 0.37$, $P = 0.709$). For soil temperatures the absolute mean error was 12 days for using mean Julian date as a predictor for peak flight, but longer at 16 days for the degree day method (Table 10.5). For the Julian date method, the largest absolute prediction error was 22 days for Lincoln 2016/2017. One should expect the degree day method, using soil temperatures considered appropriate for the subterranean stage of development, to pick up significant weather influences which would be reflected in improved prediction. However, this was not the case. The mean soil

temperature prediction was 16 days over all the sites with a large deviation of 53 days for the Lincoln 2015/2016 season. The DD accumulations calculated from soil temperatures for the Lincoln 2015/2016 site-year where much larger than all other sites, in fact 500 DD more than Manawatu that had the second highest DD total over the study period. Additionally, for the Lincoln site soil DD accumulations were higher than air DD accumulations, where the opposite was true for all other sites. Hartley and Lester (2003) also found DD accumulations around New Zealand more geographically variable than air DD accumulations. They found on average, that DD accumulations calculated from soil temperatures were 25% greater than air accumulated DDs. However, they report many sites where the DD accumulations based on soil temperatures were lower than those for air DD accumulations, as in this study. The DD for Lincoln 2015/2016 also accumulated quickly based on daily soil temperatures above threshold occurring early in the season. This means that, if the more extreme Lincoln 2015/2016 data were included or excluded from the calculation to predict peak flight, it would significantly influence the prediction for some sites. Having a large sample size of historical data that is representative of many different conditions is therefore very important. One also could speculate about the Lincoln data being almost 20 years older than that for many other sites, and what that might mean in the future. However, one would not expect the degree day method to be so wrong if it were working properly. In extreme conditions differences between the standard meteorological records and the conditions in the environment of interest. For example Zheng *et al*. (1993) suggest that the soil temperature data obtained from weather stations are usually measured in places with little or no vegetation. Furthermore, vegetation can influence greatly the surface energy balance. The environment of interest in this study is pasture which is likely to influence the soil surface energy balance.

For air temperature predictions, the difference between the Julian date method and degree day predictions was on average only two days. The variability was less for soil temperatures, with a smaller standard error and the largest deviation about 27 days, again for the Lincoln 2015/2016 site-year. Average deviations of only 12 to 16 days might encourage initial prediction of peak flight using historical averages to calculate the vulnerable period; the latter would be more accurately identified as soon as moths finish flying. For this small sample, the data suggests that even using Julian date would be useful. Of course, the alternative is to use the average optimal period for initial prediction. It might also be possible if enough historical data were accumulated to use that data to predict timing around when flights might begin, such as 1% or 5% flight, to improve advice regarding when to start putting out light traps.

The average optimal period extends from 2nd Feb to end of March for *W. copularis*. Mansfield *et al*. (2017) suggest diflubenzuron should be applied late February to early or mid-March on the West Coast. This may reflect the early abundance of *W. umbraculata* in the porina flights at several of the West

Coast sites and the complete absence of *W. cervinata* in this region. Closer study of the West Coast data using some of the methods here might suggest an earlier application for maximum impact on porina populations in some localities. But such a recommendation is not clear cut as *W. umbraculata*, a non-pest species, can occur in significant numbers early in the flight season in some West Coast sites.

While more regional data might give greater precision to the analyses completed here, even greater precision would be achieved as historical data accumulates to better characterize individual localities. Farmers of the future are likely to have their own miniaturised weather stations connected to a computer or the cloud, as well as programs for easy data collection and analysis of flight and sampling information. Analyses and procedures similar to those demonstrated here are easily programmable. Thus the process of prediction could be somewhat automated but should also be supported by visualisations of critical information to guide decision making. For example, rainfall, which is an important influence on moth flight (e.g. Mansfield *et al.* 2017), is not included in these methods but could be in some future model. An initial exploration for visualisation of weather variables over time was carried out to highlight changes in the weather surrounding porina flights. The CUSUMS technique (Manly & Mackenzie 2003) is a simple sequential analysis technique typically used for monitoring change detection. A variation of this method was used in this study to not only detect change, but to visualise when that change occurred, by how much, and for how long conditions might vary from normal. Example visualisations of accumulated standardised deviations of temperature and rainfall from average conditions, in relation to moth flight at each locality, are shown in Appendix 3. Such visualisations could be useful additions to the tool box for the modeller to help communicate maximum information about climatic conditions as well as the uncertainty surrounding predictions for forecasting. In addition, such visualisations assist communication with farmers as well as domain experts in the field. The modification used in these visualisations was the application of a smoothing technique to a weekly running average of normalised temperature and rainfall data to assist visualisation over time and smooth out daily fluctuations.

Chapter 11

General discussion

11.1 Overview of the study

Wiseana has been a major pest of pasture in New Zealand for over a century. *Wiseana* spp. are widely distributed in the southern part of the North Island and most parts of the South Island (Dugdale 1994, Barratt *et al*. 1990, Jensen & Popay 2004, Popay *et al*. 2012, Ferguson 2016, 2018). Many studies have been conducted to determine the damage caused to pasture and to develop management strategies to reduce the damage caused by this pest (Ferrell *et al*. 1974, French & Pearson 1981, Stewart & Ferguson 1992, Ferguson 2000, Ferguson & Crook 2004, Zydenbos *et al*. 2011). Studies in the early decades focused on the use of insecticides to manage this pest, in recent decades the focus has shifted to more environmentally benign strategies such as the use of endophytes, insect growth regulators, and biopesticides (Stewart & Ferguson 1992, Ferguson 2000, Jensen & Popay 2004, Easton & Fletcher 2007, Hajek *et al*. 2009, Ferguson *et al*. 2019). However, challenges are associated with their use. The development of some species within the complex have not been documented and more detailed study of development of this insect is needed to assist in the management of the pest. Because of this the objectives of this thesis were to: i) investigate the development of immature life stages of *Wiseana* spp. to adult emergence (Chapter 2); ii) investigate the feeding preferences and survivorship of porina species (Chapters 4 and 5); iii) investigate the nutritional differences between putative native hosts and exotic pasture plants (Chapter 6); iv) investigate the timing, duration and periodicity of feeding and amount eaten by porina species under controlled conditions (Chapters 4, 7 and 8); v) Develop a degree day model (Chapter 10) from novel phenology data (Chapters 3 and 9) to achieve more precise timing for implementation of control activities for *W*. *cervinata* and *W*. *copularis*.

11.2 The effect of nutrient and metabolite contents on the development and fitness of *Wiseana* spp.

Host plant quality plays a major role in larval performance and fecundity of herbivorous insects (Awmack & Leather 2002). Components of host plant quality (such as carbon, nitrogen, and defensive metabolites) directly affect larval development of herbivores. Host plant quality also affects insect reproductive strategies: egg size and quality, the allocation of resources to eggs, and the choice of oviposition sites may all be influenced by plant quality, as may egg or embryo resorption on poor-quality hosts. Nitrogen plays a role in the fecundity of herbivore insects. A good example of the importance of N in fecundity of herbivorous insects is the responses of sycamore aphid, *Drepanosiphum platanoidis* (Hemiptera: Aphididae) to changes in the quality of the phloem sap of

sycamore (*Acer pseudoplatanus*) (Dixon 1970). At the beginning of the growing season, the amino acid content of the phloem is high and individual aphids are large and highly fecund. As the sycamore leaves mature, phloem amino acids decrease and aphid reproduction ceases. When the leaves senesce, fecundity increases in response to the increased availability of amino acids in the phloem sap. The high content of N in the exotic plant *T. repens* translates to a high mean weight in larvae of *W. copularis*, *W. cervinata* and *W. umbraculata* when fed with *T. repens*. The cumulative weight of *W. copularis* also increases when they are switched from the natives *F. actae* and *P. cita* to the exotic *T. repens* high in N. The host plant *T. repens* is also high in cyclitols connected with the biogenesis of aromatic compounds (Anderson & Wolter 1966).

11.3 Niche differentiation between *Wiseana* spp.

Differences in host range are often invoked to explain the coexistence of related species of phytophagous insects in the same habitat (DeBach 1966, Rathcke 1976, Denno *et al*. 1995, Price 1997). Although differences in fundamental niches among species are often obvious in the case of specialists, they are not so in the case of extreme polyphagy, and the coexistence of several such species therefore deserves a close examination (Duyck *et al*. 2008). Many pairs of polyphagous species have proven able to coexist in the field, many of which have been recently brought into contact through unintentional introductions (White & Elson-Harris 1992, Reitz & Trumble 2002, Duyck *et al.* 2004). This study focused on the possible role of differences in another component of the ecological niche, host range, in favouring coexistence among the three *Wiseana* species present in Canterbury. The nature of the host plants can affect *Wiseana* development by direct effects on larval survival, larval development duration, larval weight, pupal weight and adult weight (Fitt 1986) but also indirectly on fecundity via pupal weight (Krainacker *et al.* 1987, 1989). We first studied the influence of three main *Wiseana* species mentioned above on survival, developmental duration and pupal weight of the three species to address different questions: (i) does host plant influence the development of the three *Wiseana* species? (ii) If so, do the different species of *Wiseana* have different host plants? (iii) Are the three species ordered differently, in terms of life-history performance, depending on host? For example, different species can be competitively dominant on different hosts although they all can exploit the same array of host plants. The goal here is to evaluate the possibility that such differences in host plant use could promote coexistence in pastures within sibling species of *Wiseana* brought by the introduction of exotic pasture plants into New Zealand. To this end, laboratory experiments and flight data were combined.

Three *Wiseana* spp. coexist sympatrically on pastures in Canterbury. Brown (1998), Barratt *et al*. (1999), Mansfield *et al*. (2017) and Richard *et al*. (2017) have shown that different *Wiseana* spp. feed

on pastures in different regions in New Zealand. Such differences could be attributed to the differing phenologies of *Wiseana* and/or to differing microhabitat preferences among the *Wiseana* species.

Flight monitoring using a light trap over two *Wiseana* flight seasons (Chapter 3) showed that *W. cervinata* and *W. copularis* were the two main species infesting pastures in Canterbury, with *W. cervinata* flying from late spring to early summer and *W. copularis* from early summer to late summer indicating a temporal differentiation between the species. The time it takes for the eggs of the three *Wiseana* species to hatch is also different for the species as temperature rises from 10℃ to 22℃ (Chapter 4, Ferguson & Crook 2004), with *W*. *cervinata* taking a shorter time to hatch which is followed by *W*. *umbraculata* and *W*. *copularis*. *Wiseana copularis* is more tolerant to higher temperatures, *W. cervinata* is more cold tolerant, while *W*. *umbraculata* is intermediate of these two species. Temperature shows differential response between the species.

An investigation of their host range (Chapter 5) showed that *W*. *copularis* had a broader host range compared to *W*. *cervinata* and interestingly, none of the plants tested was a host of *W. umbraculata*. Host preference tests done on *W*. *copularis* indicated some level of preference for the exotic pasture plant *T. repens*. Although, host preference for *W*. *cervinata* and *W*. *umbraculata* was not done, the feeding behaviour experiments (Chapter 7) of *W*. *copularis* and *W*. *cervinata* in infra-red light showed that *W*. *cervinata* fed mostly on the exotic plant *T repens*. It also showed that the two species are differentiated spatially. In such cases, modifications of the host range of certain species are sometimes observed, depending on their relative preference for one host or another (Duyck *et al*. 2008). A well-documented case is the reduction in the host range of *Ceretitis capitata* (Diptera: Tephritidae) in Hawaii, which was displaced from most cultivated hosts of the lowlands after the invasion of *Bactrocera dorsalis* (Diptera: Tephritidae) (Reitz & Trumble 2002). It is assumed that such a niche restriction results from inter-specific competition, leaving the resident species only on particular refuge hosts (Reitz & Trumble 2002).

Larvae of *W*. *copularis* constructed two horizontal burrows feeding mostly on *T*. *repens*, while *W*. *cervinata* constructed a single horizontal burrow feeding mostly on *L. perenne* × *L. multiflorum*. *Wiseana copularis* emerged earlier and more frequently than *W*. *cervinata*. Equally, pupal movement up and down an artificial burrow in response to applied stimulus, indicated that *W. cervinata* descended slightly faster than *W. copularis*.

The Ikemoto-Takai linear model was used to estimate critical parameters and it showed that T_0 and degree-days (DD) for egg-larval development of *W. copularis* was 7.5℃, 205 DD and 6.8℃, 167 DD for *W. cervinata*.

In conclusion, this study, suggests that N overall plays a critical role in the success of porina in New Zealand pastures. Further studies should clarify the role of the ancestral host plant of *Wiseana* and look for potential specific hosts of *Wiseana* in the tussock grassland. Niche partitioning is one of the basic mechanisms by which resident and introduced species may coexist (Denno *et al*. 1995, Juliano *et al*. 2002, Reitz & Trumble 2002), a consequence of which is the accumulation of species diversity after invasions (Sax *et al*. 2002, Bruno *et al*. 2005). Polyphagous species – such as *Wiseana* studied here – may have larger opportunities of invasion (in terms of fundamental niche) because they do not require specific hosts; however, they are also more exposed to inter-specific competition. More studies on invasions by both specialist and generalist taxa are needed to test this general prediction. In addition, other mechanisms (not depending on a potential niche partitioning) could promote co-existence, such as aggregative spatial and temporal distribution (Shorrocks *et al.* 1984, Wertheim *et al.* 2000) and competition colonisation trade-offs (Tilman 1994). The coexistence of the three closely related *Wiseana* spp. in Canterbury was due to distinct host-plant and micro habitat preferences.

11.4 Recommendations for future research

- Compare host plant responses for porina populations sourced from native vs. exotic habitats.
- Detailed genetic analysis to look at population mixing between habitats and/or genetic differentiation between such populations.
- Studies of the interactions between *Wiseana* spp. and endophyte ryegrass varieties.
- Development studies were done on only three of the seven *Wiseana* species, a similar study should be done on *W. mimica*, *W. jacosa*, and *W. signata*. Also more native plant species should be used so that the original native plant host(s) can be determined.
- The detailed life cycle parameters could be used in a more detailed population model that can show projections and possibilities of population dynamics under different environmental conditions.
- Studies on the fitness response to host-shift of *Wiseana* species from the exotics to the natives' shoud be carried out.
- Nitrogen was the main nutrient linked with positive host plant responses, so protein analysis on these host plants should be done.

- To improve on the model, better developmental parameters need to be included in the model and to achieve that more detailed development data from *Wiseana* spp., particularly on the larval-pupal stage and on pupal-adult emergence should be collected.

- Despite the potential of the DD method for forecasting, particularly the first flight, and pest management, further research on *Wiseana* development at varying temperatures is required to establish more precisely DD requirements in the field. The development and refinement of effective forecasting systems requires large amounts of both accurate long-term data on pest incidence and climate variables, collected over a wide geographic area.

- Validation of the model using flight data from other sites in New Zealand should be done.

- The roles of native plant defences, intensive cultivation of pastures, and separation of porina from their co-evolved natural enemies should be investigated.

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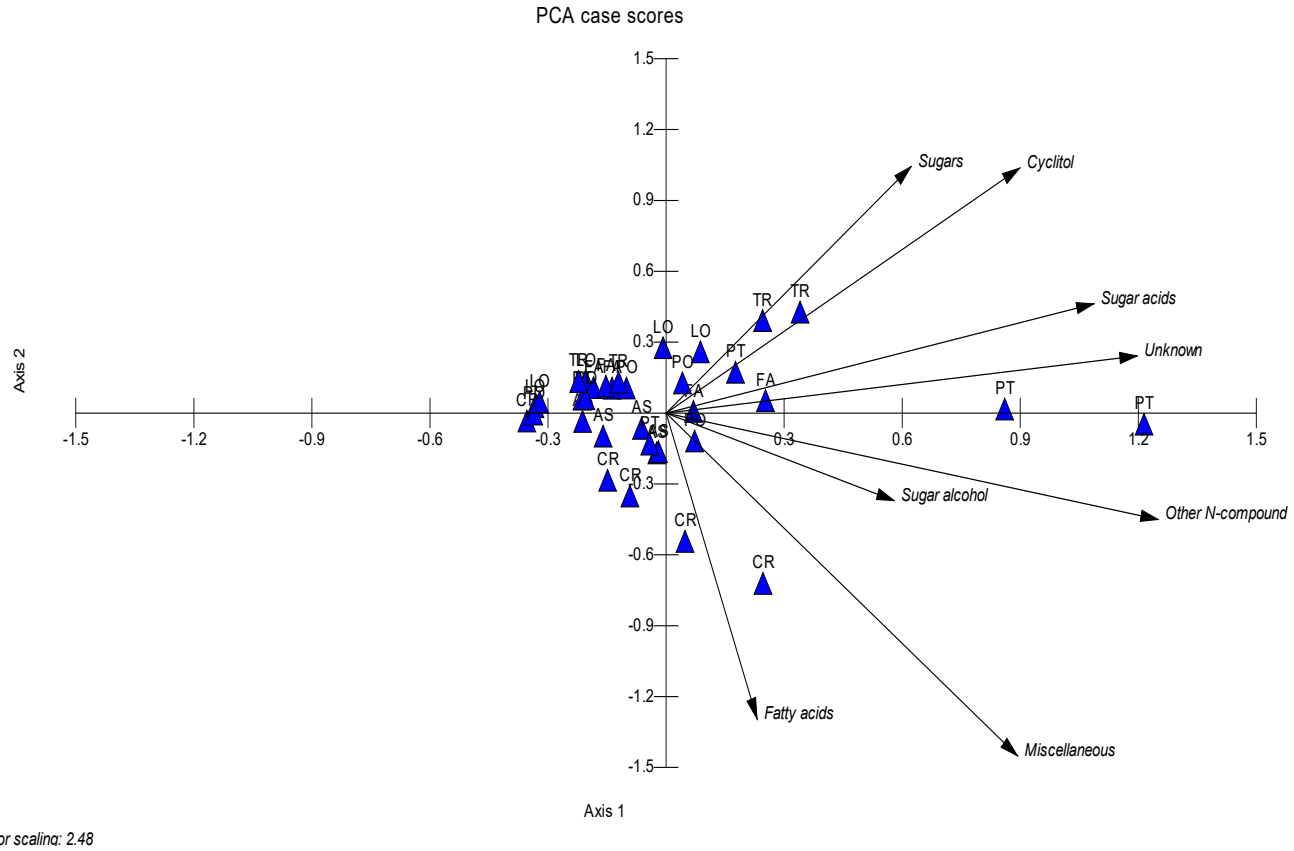
Appendix A

Quantity eaten by *W. copularis* larvae offered choices of exotic and native plants and analysed using a paired t-test

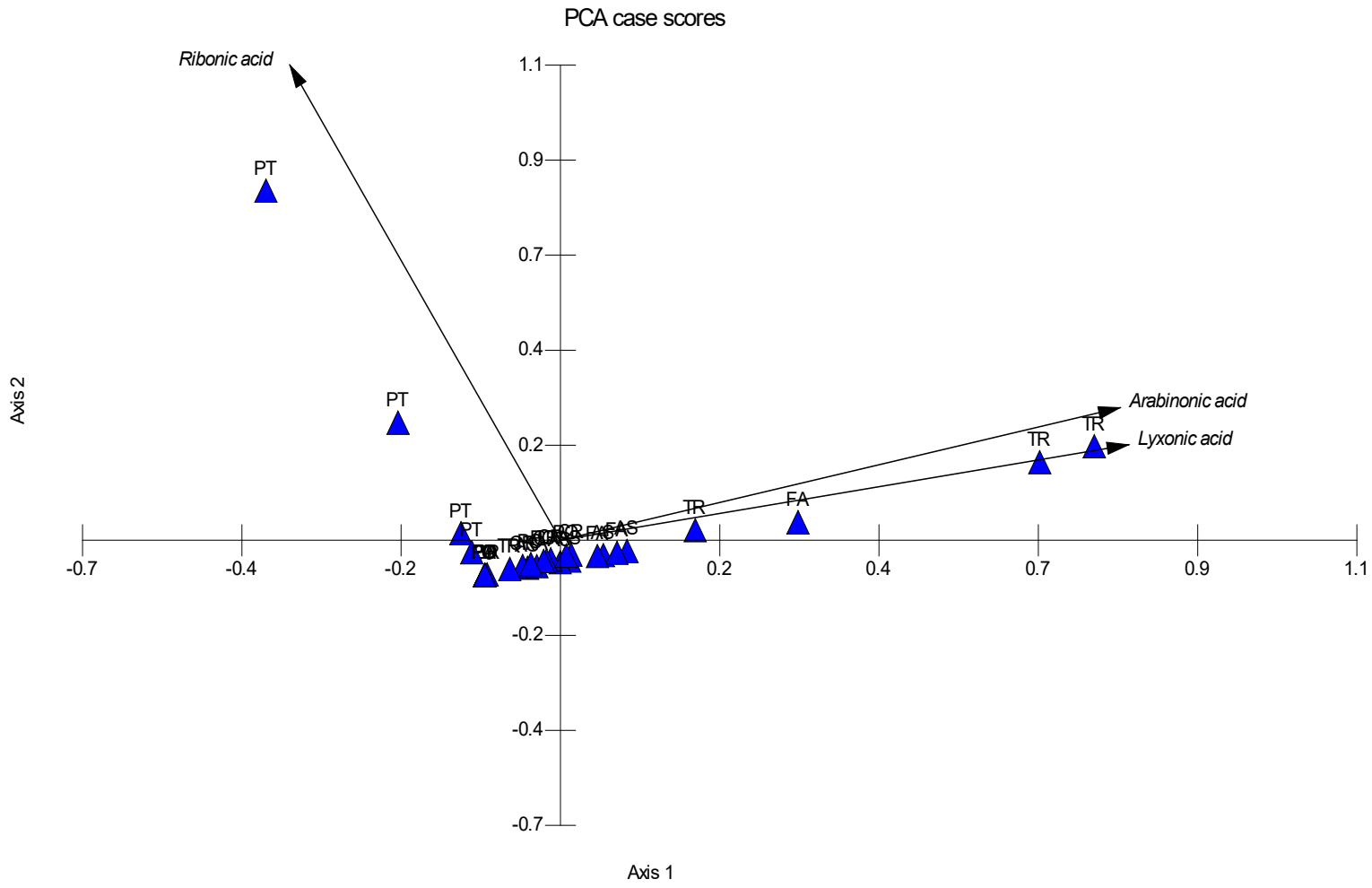
Exotic plants	**Mean (mg)**	**Native plants**	**Mean (mg)**	***n***	**t**	**df**	**P**
T. repens	3.09 ± 0.57	*P. tenax*	0.77 ± 0.46	11	3.63	10	0.005
T. repens	1.91 ± 0.88	*A. squarrosa*	0.00 ± 0.00	11	2.18	10	0.055
T. repens	2.05 ± 0.69	*F. actae*	0.41 ± 0.28	11	2.93	10	0.207
T. repens	1.41 ± 0.58	*C. rubra*	0.66 ± 1.06	11	0.47	10	0.286
T. repens	0.91 ± 0.46	*P. cita*	0.73 ± 0.27	11	1.30	10	0.774
Exotic plants	**Mean (mg)**	**Native plants**	**Mean (mg)**	***n***	**t**	**df**	**P**
L. perenne × *L. multiflorum*	0.50 ± 0.24	*P. tenax*	0.21 ± 0.12	11	0.21	10	0.347
L. perenne × *L. multiflorum*	0.57 ± 0.30	*A. squarrosa*	0.71 ± 0.43	11	1.31	10	0.814
L. perenne × *L. multiflorum*	0.36 ± 0.15	*F. actae*	0.59 ± 0.54	11	0.09	10	0.710
L. perenne × *L. multiflorum*	0.36 ± 0.15	*C. rubra*	1.64 ± 0.99	11	-1.56	10	0.212
L. perenne × *L. multiflorum*	0.18 ± 0.12	*P. cita*	0.09 ± 0.09	11	0.78	10	0.588
Exotic plant	**Mean (mg)**	**Exotic plant**	**Mean (mg)**	***n***	**t**	**df**	**P**
T. repens	30.10 ± 14.00	*L. perenne* × *L. multiflorum*	12.10 ± 4.41	11	1.67	10	0.126

Appendix B



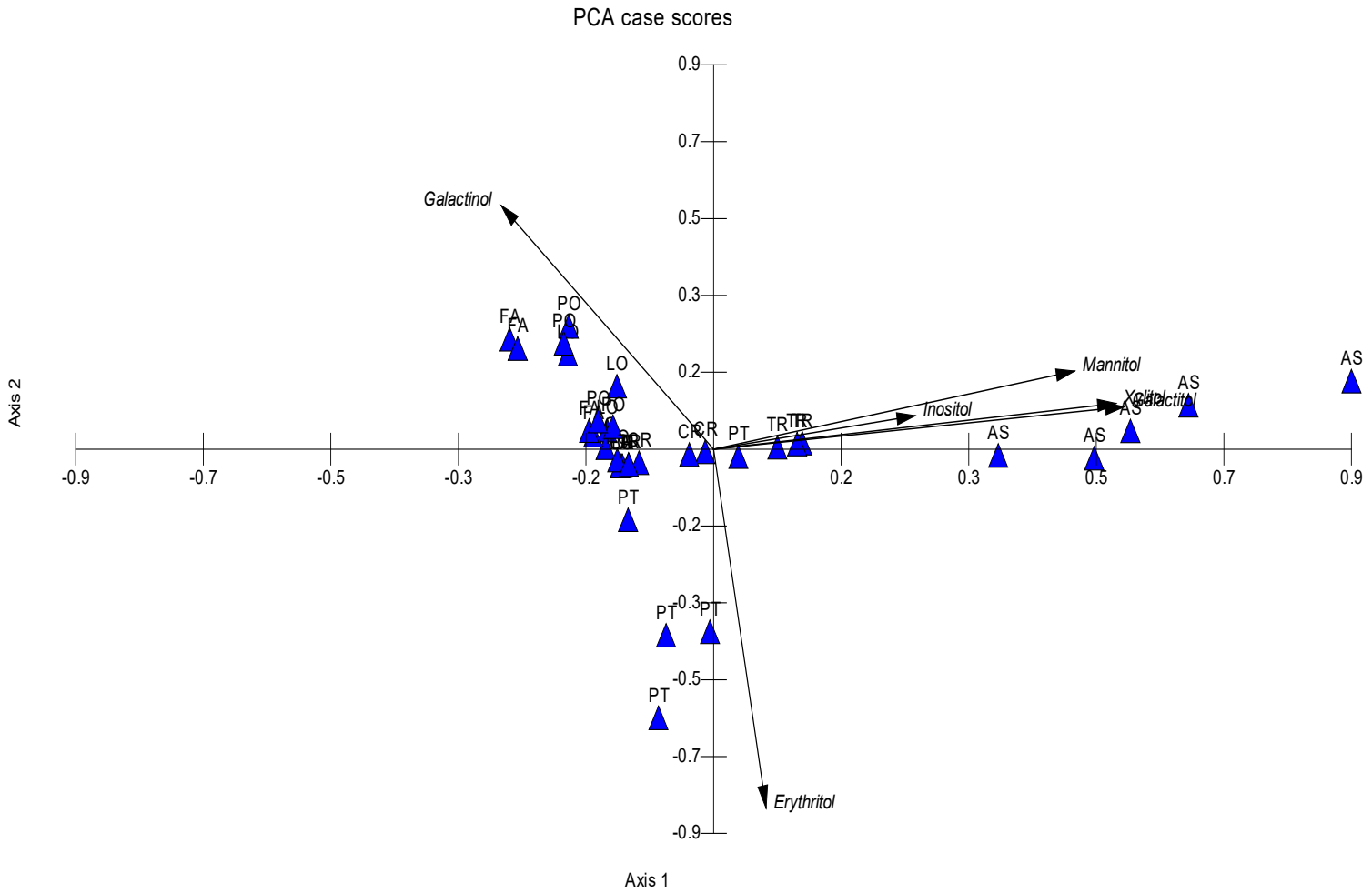
Vector scaling: 2.48

Biplot of principal component (PC) analysis of amino acids, cyclitols, fatty acids, organic acids, other N-compounds, phytosterols, sugars, sugar acids, sugar alcohols, miscellaneous and unknown compounds of samples from all seven plants, PC 1 = 40%, PC 2 = 21%.

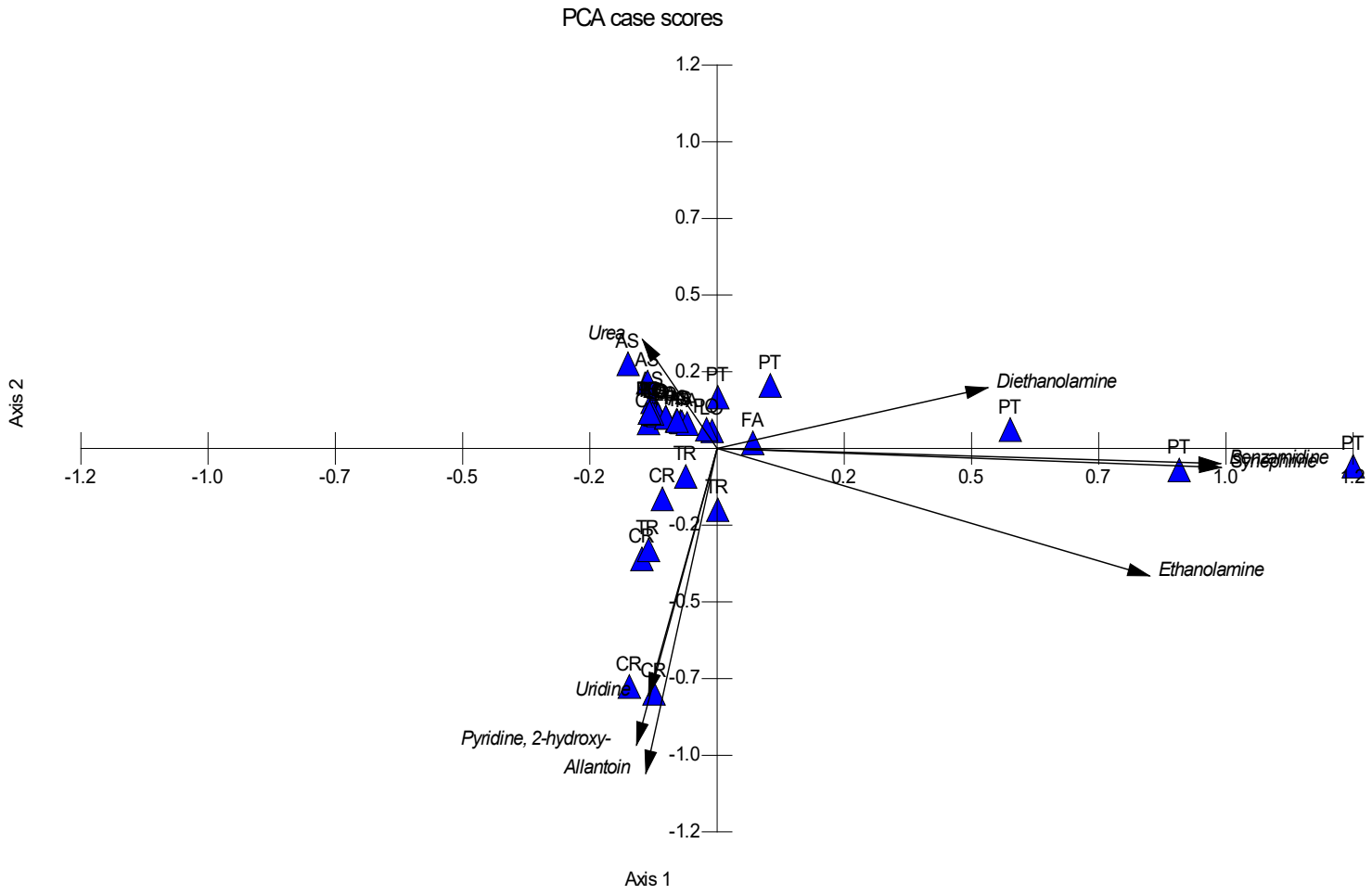


Vector scaling: 1.18

Biplot of PC analysis of sugar acids of samples from all seven plants, PC 1 = 52%, PC 2 = 31%.

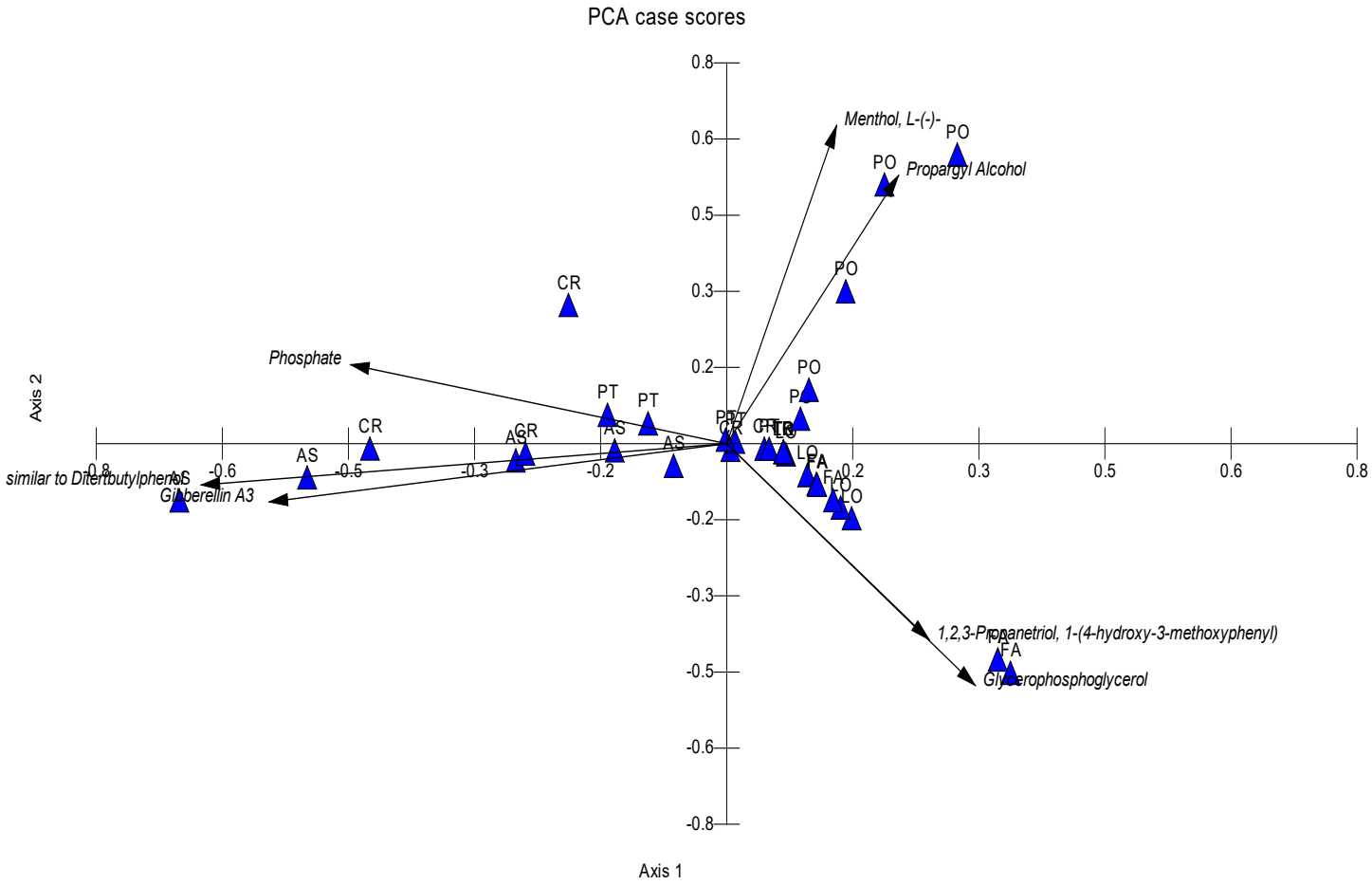


Biplot of PC analysis of sugar alcohols of samples from all seven host plants.

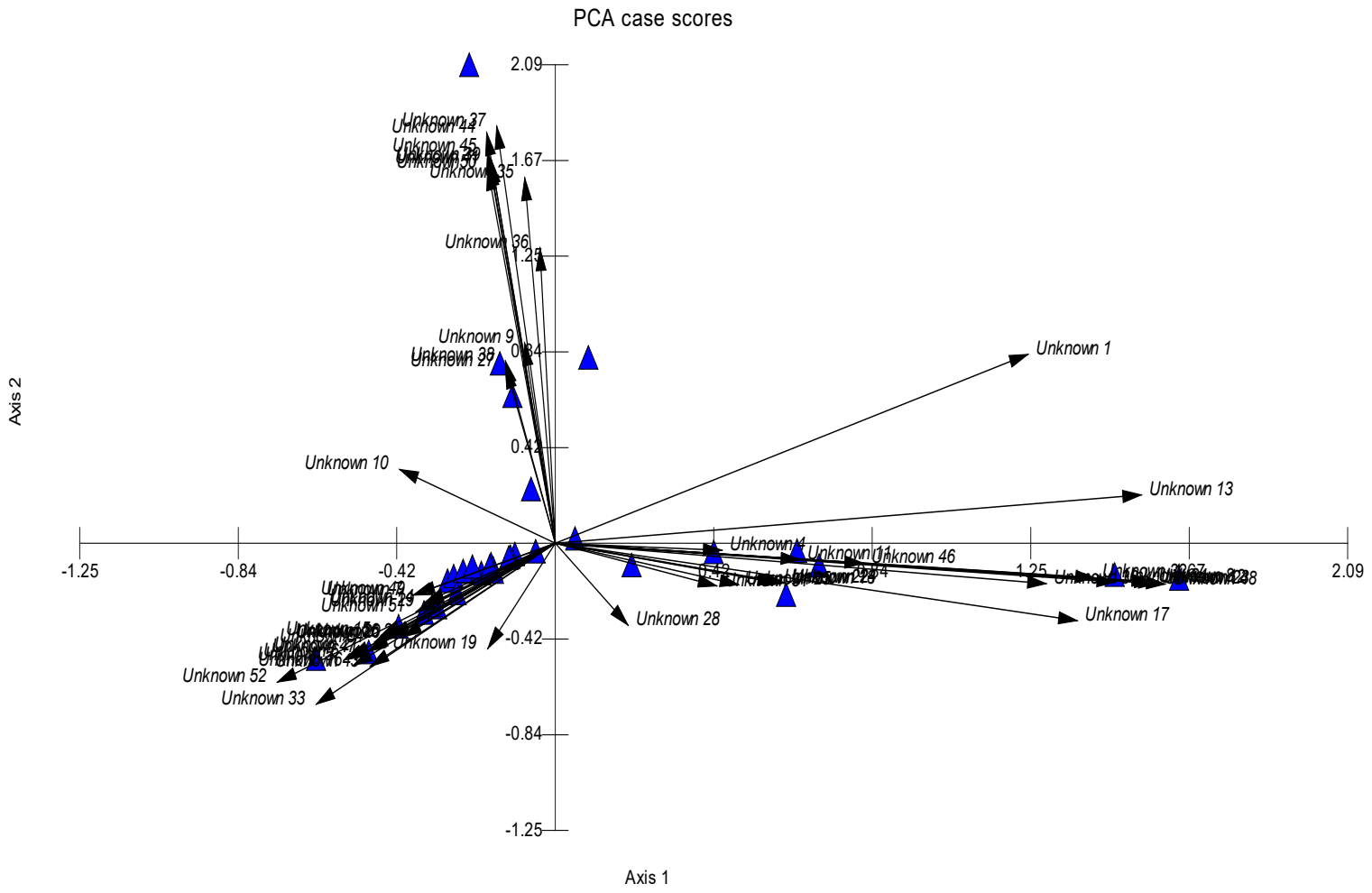


Vector scaling: 1.70

Biplot of PC analysis of other N-compounds of samples from all seven plants. PC 1 = 37%, PC 2 = 23%.



Biplot of PC analysis of miscellaneous compounds of samples from all seven plants. PC 1 = 26%, PC 2 = 21%.



Vector scaling: 5.41

Biplot of PC analysis of unknown compounds of samples from all seven plants. PC 1 = 15%, PC 2 = 14%.

Mean relative abundances of primary metabolites (µg) identified by GC-MS in the shoot of the plants. Identities were either verified by comparison with standards, with the help of the online Glom database (GMD) using spectra and kovats indices or could not be identified.

No	Metabolites (µg)	Retention indices	*T. repens*	*L. perenne × L. multiflorum*	*F. actae*	*C. rubra*	*P. cita*	*A. squarrosa*	*P. tenax*
	Amino acids								
1	Alanine	1107	1211		2040	4694	760	765	728
2	Norvaline	1177						418	
3	Valine	1178/1223	472	1354	2243	1891	482	809	
4	Leucine	1280	251		539	704		373	
5	Isoleucine	1303/1567	804				154	767	
6	Proline	1305/1307/1595				2148	2465		
7	Threonine	1305/1401	906		2201	2876	1255	432	1999
8	Glycine	1315	302	837	990	909	460	294	742
9	Serine	1373	2270		4463	8361	848	390	1818
10	Asparagine	1511/1609/1626 1640/1649/1688 1693/1978*	25787		100223	115786	1709	6149	25147
11	Aspartic acid	1536	4810		6645	9872		2487	5269
12	Pyroglutamic acid	1538					9412		
13	Glutamic acid	1635	5438	1492	7348	14709	1314	2704	4176
14	Phenylalanine	1639						667	
15	Glutamine	1790	932		4977	9352	1109	466	
16	Adenine	1883	455					1233	
17	Lysine	1938	707		5414	2938		277	
18	Tyrosine	1957	342		666	532		825	
	Cyclitols								
1	Shikimic acid	1832*		3070	6203	881	1100		3615
2	Pinitol	1865	56475						
3	Quinic acid	1892/3171/3228		11631	23262	2139	22463	5438	28484

4	Ononitol	1987	57839						
	Fatty acids								
1	Hexadecanoic acid	2049				2362			
2	Octadecadienoic acid	2216/2219/2224	464		3815	6105	2340	319	678
3	Octadecanoic acid	2249					1254		
	Miscellaneous compounds								
1	Propargyl alcohol						5339		
2	Phosphate	1285				28413		13802	25057
3	Menthol, L-(-)-	1523				204	1158		
4	similar to Ditertbutylphenol	1553				99		120	
5	Glycerophosphoglycerol	2234		547	1302	111			
6	1,2,3-Propanetriol, 1-(4-hydroxy-3-ethoxyphenyl)	3012		284	5050				
7	Gibberellin A3	3447						300	
	Organic acids								
1	Butanoic acid	1541	4996	5285	6883	2585	2825	2013	2401
2	Hexanoic acid	1044							647
3	Malonic acid	1213*	1943		770		2285		
4	Succinic acid	1320	1715	1024	827	838	1325	358	812
5	Glyceric acid	1344	24552			194		405	349
6	Fumaric acid	1353	543		620		953	187	
7	Dihydroxymalonic acid	1476	5366						
8	Malic acid, 2 methyl	1487						8481	
9	Malic acid	1505*	60502	59140	37739	7651	52690	26601	61071
10	Erythronic acid	1578	5175						3048
11	Glutaric acid, 2-oxo-	1593/1739	573				351		
12	Maleic acid	1697					472		
13	Aconitic acid	1767/1774				50172			
14	Gluconic acid	1839/1875				675		1477	1985

15	Citric acid	1845/1928*	11926	28691	21311	12453	8839	491	18092
16	Tetradecanoic acid	1854					966		239
17	Kojic acid	1870				979			
18	Dehydroascorbic acid	1877		429					
19	Gulonic acid	2001/2054	120					83	877
20	Caffeic acid	2155			1594				
	Other N-compounds								
1	Benzamidine								622
2	Diethanolamine								981
3	Pyridine, 2-hydroxy-	1038	195			128			
4	Synephrine	1129							14464
5	Urea	1247						153	
6	Ethanolamine	1274	2005	931	2030	1642	824	827	3938
7	Allantoin	1913				2752			
8	Uridine	2481				182			
	Phytosterols								
1	Campesterol	3293	465	5692		347			
2	Stigmasterol	3324	517					236	488
3	Beta-Sitosterol	3384	2095	1614	2554	4256	1734	992	2286
	Sugars								
1	Xylose	1677	348						
2	Ribose	1707	1023			743	852		5815
3	Fructose	1836/1849/1910 /1922*	33949	39829	33948	7768	128553		87121
4	Tagatose	1907			41218	4816		10089	
5	Sorbose	1913/1918*	29852	82653		4091		79976	100678
6	Glucopyranoside, 1-O-methyl-, beta-D-	1932/2564/2567	59702		475				
7	Galactose	1933/1951/2152*	15429	169830	49520	17397	132780	12232	94846

8	Glucose	1936*			71944				
9	Glucopyranose, D-	2017	2392			10381	5621		1239
10	similar to Glycerolaldopyranosid	2359	375			351	426	358	
11	Glucose-6-phosphate	2372				230			
12	Glucoheptose	2413						517	
13	Ketose	2700/3501	96822	162642	195388			57915	
14	Sucrose	2705*					121528		135168
15	beta-D-Fructofuranosyl-(2,1)-beta-D- Fructofuranose	2757		2195	3358				
16	Maltotriose	2771/2908		1428		168		81	5920
17	Melibiose	2800				785			
18	Trehalose	2803	552			113	1788	460	1841
19	Melezitose	3277						1314	
20	Raffinose	3484/3540*		2489	3210		2066	883	480
	Sugar acids								
1	Threonic acid	1587*		512			358		
2	Lyxonic acid	1782	614		520			395	
3	Arabinonic acid	1797	897			222	163		
4	Ribonic acid	1798					1033		
	Sugar alcohols								
1	Erythritol	1530						132	557
2	Xylitol	1724	360					563	
3	Mannitol	1878						762	
4	Inositol	1926/2064/2125	8236	8223		12857	10262	18148	16963
5	Galactitol	1964						19765	
6	Galactinol	3060		2794	3824		4042	93	664
	Unknown compounds								
1	Unknown 1		889				1018		965

2	Unknown 2			278				
3	Unknown 3	1127					8989	
4	Unknown 4	1141						3194
5	Unknown 5	1487				308		
6	Unknown 6	1564				1002		
7	Unknown 7	1574					403	
8	Unknown 8	1606	2500					
9	Unknown 9	1771					219	1022
10	Unknown 10	1783				898		1027
11	Unknown 11	1828	361					
12	Unknown 12	1837/1852	1663					
13	Unknown 13	1915	1650				1207	747
14	Unknown 14	1915			10512			
15	Unknown 15	1991		3476	1034	710	1006	906
16	Unknown 16	2019		3476	1034	710	1006	906
17	Unknown 17	2034	2500			887		
18	Unknown 18	2057					349	
19	Unknown 19	2068	625	2796		132		
20	Unknown 20	2080				1864		
21	Unknown 21	2085				334		
22	Unknown 22	2145					434	
23	Unknown 23	2153	416					
24	Unknown 24	2179					267	
25	Unknown 25	2237					1428	
26	Unknown 26	2252				684		
27	Unknown 27	2285						432
28	Unknown 28	2347			2561			
29	Unknown 29	2358			934			
30	Unknown 30	2362	253				383	

31	Unknown 31	2375					294		
32	Unknown 32	2391	1293						
33	Unknown 33	2419			454	1694	403	799	
34	Unknown 34	2421	887						
35	Unknown 35	2441							21943
36	Unknown 36	2450							26577
37	Unknown 37	2545							659
38	Unknown 38	2557							194
39	Unknown 39	2719							1828
40	Unknown 40	2727	428		1676		249		414
41	Unknown 41	2734							425
42	Unknown 42	2763					852		
43	Unknown 43	2774		5315	3619				
44	Unknown 44	2781							6424
45	Unknown 45	2791							1749
46	Unknown 46	2819	1033						
47	Unknown 47	2829		1294	1616				
48	Unknown 48	2873	321						
49	Unknown 49	2904						690	
50	Unknown 50	2940							390
51	Unknown 51	3083			1999				
52	Unknown 52	3201		1303		1671		3143	
53	Unknown 53	3205			2551		2307		597
54	Unknown 54	3237				2966			

* Metabolites confirmed using pure samples.